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Analysis of the Mechanisms of Macrophage Activation in Fish

by

James Lee Stafford ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Physiology and Cell Biology

Department of Biological Sciences

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Sept. 4/2003

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University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Analysis of the Mechanisms of Macrophage Activation in Fish" submitted by James Lee Stafford in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiology and Cell Biology.

Dr. Miodrag Belosevic
(Supervisor)

Dr. Jeff Goldberg
(Committee member)

Dr. Dennis Kunimoto
(Committee Member)

Dr. John Samuel
(Internal Examiner)

Sept. 3/2003

Dr. Norman Miller
(External Examiner)

TO MY FAMILY AND FRIENDS:

INDIVIDUAL ACCOMPLISHMENTS WOULD NEVER BE ACHIEVED
WITHOUT THE ENCOURAGEMENT AND SUPPORT OF OTHERS.
I THANK YOU ALL

ABSTRACT

The most important phagocytic cell in the vertebrate body is the macrophage, and cells functionally reminiscent of the vertebrate macrophage are present in virtually all metazoan organisms, attesting to the importance of phagocytosis in host defense in all multi-cellular organisms. Macrophages play a pivotal role in the detection of pathogenic microorganisms and in the ensuing effector phases responsible for elimination of infectious agents. Characterization of the native factors that modulate macrophage functions in mammals has resulted in the identification of wide variety of cytokines and chemokines. Exploring the functional roles of these proteins, identification of their receptors, and detailed analysis of their effects on macrophage functions has contributed significantly to our understanding of cellular immune processes in higher vertebrates. Conversely, we know very little about the native factors that modulate these mechanisms in lower vertebrates including fish. As such, the majority of teleost 'cytokine-like' factors have been identified using molecular cloning techniques with very few studies designed to understand the functional roles of these molecules. The main objective of my thesis was to identify the native factors that modulate goldfish macrophage activation using a combination of molecular techniques and the more traditional biochemical approaches.

Major findings reported in this thesis are that: 1) primary macrophage-like cultures could be established from carp and primary monocyte-like cultures could be established from rainbow trout using protocols originally designed to cultivate primary goldfish macrophages; 2) goldfish macrophage antimicrobial functions were significantly inhibited by both endogenous and exogenous factors;

3) one of the major goldfish macrophage activating factors, purified from mitogen-stimulated goldfish kidney leukocyte supernatants, was transferrin cleavage products; 4) production of recombinant transferrin cleavage products indicated that transferrin can activate both fish and mammalian macrophages, suggesting that this is a conserved mechanism of macrophage activation; and 5) goldfish macrophages express Toll-like receptors (TLRs), which belong to a family of highly conserved innate immune receptors. Taken together these findings provided the basis for the development of a model of immune activation in fish mediated by a non-cytokine host protein.

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LIST OF ABBREVIATIONS

AMP:	ampicillin
AP:	alkaline phosphatase
4-AP:	4-aminopyridine
BCIP:	5-bromo-4-chloro-3-indolyl phosphate, p-touluidine salt
BMDM:	bone marrow-derived macrophages
β-ME:	beta-mercaptoethanol
bp:	base pairs
CARB:	carbenicillin
CCM:	cell-conditioned medium
C-FPLC:	chromatofocusing-fast performance liquid chromatography
ConA:	concanavalin A
CR3:	complement receptor 3
CS:	carp serum
DEAE:	diethylaminoethyl
DHR:	dihydrorhodamine
DMSO:	dimethyl sulfoxide
EST:	expressed sequence tag
FBS:	fetal bovine serum
FCS:	fetal calf serum
FPLC:	fast performance liquid chromatography
GMCL:	goldfish macrophage cell line
GM-CSF:	granulocyte/macrophage colony stimulating factor
GP-FPLC:	gel permeation fast performance liquid chromatography
GSH:	reduced glutathione
GSP:	gene specific primer
GSSG:	oxidized glutathione
HBSS:	Hanks balanced salt solution
HSP:	heat-shock protein
IFN:	interferon
IL:	interleukin
IPTG:	isopropyl β-D-thiogalactoside
iNOS:	inducible nitric oxide synthase
IVDKM:	*in vitro* derived kidney macrophages
kD:	kilodaltons
LAL:	limulus amebocyte lysate
LB:	Luria-Bertani
LPG:	Lipophosphoglycan
LPS:	lipopolysaccharide
LRR:	leucine-rich repeats
LTA:	lipoteichoic acid
MAF:	macrophage activating factor(s)
MALDI-TOF:	matrix-associated laser desorption/ionization-time of flight

MBP:	mannose-binding protein
M-CSF:	macrophage colony stimulating factor
MDF:	macrophage deactivating factor(s)
MGF:	macrophage growth factor(s)
MLR:	mixed lymphocyte reaction
MR:	mannose receptor
MW:	molecular weight
MWCO:	molecular weight cut-off
NBT:	nitro blue tetrazolium
NiNTA:	nickel-nitrilotriacetic acid
NO:	nitric oxide
NRAMP:	natural resistance associated macrophage protein
O.D.:	optical density
PAMPs:	pathogen associated molecular patterns
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PEG:	polyethylene glycol
pI:	isoelectric point
PMA:	phorbol myristate acetate
PMB:	polymixin B
PMSF:	phenyl methyl sulforyl fluoride
PRR:	pattern recognition receptor
RACE:	rapid amplification of cDNA ends
RFU:	relative fluorescence units
RNI:	reactive nitrogen intermediates
ROI:	reactive oxygen intermediates
RT-PCR:	reverse transcriptase polymerase chain reaction
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE:	salt elution
SP-A:	lung surfactant protein-A
SSH:	suppressive subtractive hybridization
SR:	scavenger receptor
TEA:	tetraethyl ammonium chloride
TGFβ:	transforming growth factor beta
TIR:	Toll/Interleukin-1 response (TIR) domain
TLR:	Toll-like receptor
TNFα:	tumor necrosis factor alpha
T-PKM:	trout primary kidney monocytes
TOPO:	topoisomerase
V_m:	plasma membrane potential
8C2:	polyclonal anti-nitric oxide-inducing factor antibody
9AG7:	polyclonal anti-carp transferrin antibody

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW[1]

1.1 Introduction

One of the fundamental roles of the immune system is to recognize self from non-self; discriminating the finite structure of foreign molecules from the diverse array of molecular patterns and complexities intrinsic to the host. Non-self recognition mechanisms appear to be an inherent prerequisite for the survival of any living organism, and are present even within the simplest life forms, the mycoplasms, which contain enzymes that have evolved to recognize and degrade invasive foreign genetic material (i.e., bacteriophages) [1]. Many diverse eukaryotic non-self recognition mechanisms have evolved to discriminate foreign proteins, carbohydrates, lipids, and invasive nucleic acids (viruses and virions). These non-self recognition systems are intricately coupled to host defensive mechanisms, such as phagocytosis, which mediate the destruction and eventual elimination of invasive pathogenic microorganisms.

Phagocytosis is the primordial defense mechanism of all metazoan organisms. In fact, it has even been suggested that phagocytosis represents a rudimentary innate defense mechanism in the most primitive eukaryotic kingdom, the Protozoa [2]. Haeckel first described phagocytosis in 1862, using blood cells from the gastropod *Tethys spp.* [3], but its importance as a defense mechanism was not realized until 1882, by Elie Metchnikoff [3]. Metchnikoff's classic experiments involved impaling a starfish larva with a rose thorn. He noticed that amoeba-like cells invaded the damaged tissues, and surrounded the thorn; an apparent attempt by the starfish larva to defend itself against this foreign object. Metchnikoff was cognizant of the similarities between this primitive cellular defense mechanism in invertebrates and the inflammatory processes observed in higher vertebrates. This simple observation provided the theoretical foundation for elucidating the mechanisms that initiate innate cell-mediated immunity in vertebrates and in characterizing the roles that phagocytic cells play in host defense against infectious disease.

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The most important phagocytic cell in the vertebrate body is the macrophage, and cells functionally reminiscent of the vertebrate macrophage are present in virtually all metazoan organisms, attesting to the importance of phagocytosis in host defense in all multi-cellular organisms. Macrophages play a pivotal role in the detection of pathogenic microorganisms and in the ensuing effector phases in eliminating the infectious agent. Pathogen-associated molecular patterns (PAMPs) on the surface of pathogenic microorganisms are recognized by pattern recognition receptors (PRR) on macrophages, facilitating the phagocytic process and initiating subsequent killing mechanisms [4-6].

The ubiquitous distribution of macrophages within the vertebrate body ensures the continuous *surveillance* of host tissues for foreign invaders. In many cases, it is the macrophage (or lineage-related cells such as dendritic cells) that provides the first line of cell-mediated host defense against pathogens. Foreign microorganisms are rapidly phagocytosed by macrophages, and are destroyed by lysosomal enzymes released into the phagosome, or by toxic reactive intermediates formed by activated macrophages. In addition, macrophages possess a number of nutrient deprivation mechanisms that are employed to *starve* phagocytosed pathogens of essential micro-nutrients. The fundamental roles of macrophages in host defense are to recognize foreign invaders, limit the initial dissemination and/or growth of infectious organisms, and to modulate ensuing immunological reactions.

1.2 Objectives of the Thesis

Characterization of the native factors that modulate macrophage functions in mammals has resulted in the identification of a wide variety of cytokines and chemokines. Exploring the functional roles of these proteins, identification of their receptors, and detailed analysis of their effects on macrophage functions has contributed significantly to our understanding of cellular immune processes in higher vertebrates. Conversely, we know relatively little about the native factors that modulate these mechanisms in lower vertebrates including fish. As such, the majority of teleost 'cytokine-like' factors have been identified using molecular cloning techniques with very few studies designed to understand the functional roles of these molecules. The main objective of my thesis was to identify the native factors that modulate goldfish macrophage activation using both biochemical and molecular approaches. The specific aims of my research were:

a) to adapt the cultivation procedures originally used to isolate *in vitro*-derived primary goldfish kidney macrophages for the isolation of functional primary kidney-derived macrophage- and monocyte-like cells from two other economically important fish species (i.e. carp and rainbow trout).

b) to biochemically and functionally characterize both endogenous (leukocyte-derived) and exogenous (pharmological blockers of potassium channels) factors that exhibited macrophage deactivation activities.

c) to characterize a nitric oxide-inducing factor(s) purified from mitogen-stimulated goldfish kidney leukocyte supernatants.

d) to clone and express transferrin and test the ability of the recombinant protein to induce nitric oxide response in macrophages.

e) to develop a model of macrophage activation mediated by transferrin cleavage products and highly conserved innate immune receptors (i.e. Toll-like receptors) and to discuss the potential conservation of this mechanism.

1.3 Outline of thesis

In the first chapter, I review the current literature in regards to macrophage-mediated innate host defence mechanism in mammals and fish. This chapter focuses on how macrophages detect foreign invaders and the subsequent induction of antimicrobial mechanisms required for preventing the dissemination of pathogens by reviewing both the mammalian and teleost literature. Chapter 2 contains a detailed description of the Materials and Methods used throughout the thesis. Chapter 3 describes results of *in vitro* cultivation techniques used to obtain primary macrophage cultures from different fish species. Specifically, in this chapter, I demonstrate that the original cultivation procedures used to isolated goldfish *in vitro*-derived kidney macrophages can be used to isolate functional monocyte-like cells from rainbow trout and functional monocyte-and macrophage-like cells from carp. In Chapter 4, I present the results of studies designed to characterize both endogenous and exogenous factors that exhibited potent macrophage deactivation activities. The fifth chapter demonstrates that a major goldfish macrophage nitric oxide-inducing factor found in mitogen-stimulated goldfish kidney leukocyte supernatants is transferrin cleavage products. Furthermore, both mammalian (i.e. bovine) and fish (i.e. carp and goldfish) transferrins appear to be capable of inducing this response. Chapter 6 focuses on the cloning and expression of recombinant goldfish transferrin using both prokaryotic and eukaryotic expression systems. Results in this chapter demonstrate that recombinant goldfish transferrin N- and C-lobes, expressed in *E. coli*, significantly induced nitric oxide production in goldfish and murine macrophages. The cloning and gene expression analysis of one of the first discovered teleost Toll-like receptors is the focus of Chapter 7. Finally, in Chaper 8, I present a model of macrophage activation in teleost, mediated by cleaved transferrin products. In this chapter I suggest that this is a highly conserved and perhaps primitive mechanism for the induction of innate immune responses in both vertebrates and invertebrates.

1.4 Literature Review

1.4.1 Overview

The roles that macrophages play in mediating innate host defense in mammals and teleost is reviewed. I first describe how macrophages recognize and bind foreign agents using a repetoire of innate immune receptors known as pattern recognition receptors (PRR). This is then followed by a description of the induction of antimicrobial responses required for the elimination of pathogens. Specifically, I review the mechanisms used by macrophages to restrict the growth of pathogens, which include deprivation of essential nutrients (i.e. sequestration of available iron and degredation of tryptophan), and the induction of the respiratory burst and nitric oxide production pathways as inducible antimicrobial responses in macrophages. Since much of the information collected to date was derived from comparative studies relating teleost macrophage function to the well characterized mammalian macrophages, it is imperative to first highlight these mechanisms that have been described in higher vertebrates (i.e. mammals) and then focus on reviewing the literature that described host defense mechanisms of fish.

1.5 Recognition of infectious agents by macrophages

How macrophages *sense* a foreign molecule within the context of a molecularly complex host environment is extraordinary. The possible number of foreign molecular configurations on a diverse assemblage of pathogenic microorganisms is immense. For most metazoan organisms the evolution of cellular mechanisms for recognition of all possible foreign molecule conformations is limited, in most cases, by the size of the genome. Although vertebrates have evolved adaptive immune responses to recognize large numbers of foreign molecules (i.e. T-cell receptors and antibodies), these same mechanisms appear to be absent from invertebrates, and as such, greater than 99% of all multi-cellular life forms rely on non-self *innate* immune mechanism to recognize foreign molecules. The extensive diversity of foreign molecular structures, accompanied by limited genome sizes, necessitated the evolution of non-self discriminatory mechanisms that recognized unique molecular configurations on *classes* of microorganisms. These unique foreign molecular configurations are also known as pathogen-associated molecular patterns (PAMPs) [5-6]. PAMPs all have the common features of being unique to a group of pathogens, are essential for survival, and are relatively invariant in their basic structure. Specific examples of PAMPs include lipopolysaccharide (LPS; gram negative bacteria), lipoarbinomannan (mycobacteria), lipoteichoic acids (LTA; gram positive bacteria), mannans (yeast), and double stranded RNA (viruses).

PAMPs are recognized by pattern recognition receptors (PRRs) found on the surface of immune cells including macrophages [6-7]. These innate immune receptors are a germ-line encoded group of receptors with a genetically pre-determined specificity that are highly conserved among different organisms. Pattern recognition receptors have distinctive ligand-binding properties for specific classes of PAMPs, but are less discriminatory towards the subtle differences in fine molecular structure within that class of PAMPs. For example, CD14, a PRR for bacterial LPS, can bind to LPS from a diverse range of microorganisms, even though the fine structure of LPS can vary among different bacterial species [8]. In addtion, both Toll and Toll-like receptors (TLRs) are believed to not only bind foreign material, but are also capable of binding endogenous proteins (i.e. recognition of cleaved Spatzle by *Drosophila* Toll and recognition of fibrinogen and fibronectin by TLR-4) [9-11].

Examples of some of the major proteins involved in microbial pattern recognition include mannose-binding proteins, mannose receptor, scavenger receptor, CD14, and TLRs. Functionally, PRRs can be divided into three classes; 1) secreted

PRRs, which usually function as opsonins or activators of complement, 2) endocytic PRRs, which function in pathogen binding and phagocytosis, and 3) signaling PRRs that activate gene transcriptional mechanisms that lead to cellular activation. This portion of the review focuses on these PRR, which are found on the surface of macrophages and I will discuss their roles in pathogen recognition/binding.

1.5.1 Lectins

Lectins play an important role in the recognition of pathogens and serve multiple functions in the immune system, including cell adhesion, recruitment, differentiation and activation [12-13]. Many of the lectins involved in the recognition and neutralization of pathogens are members of the C-type or calcium-dependent animal lectin families [14]. Within this family are two major groups of PRRs that are important components of the innate immune response; the collectins and the mannose receptor. Collectins are large soluble proteins that mediate pathogen neutralization through the complement pathway and include pulmonary surfactant proteins (SP-A and SP-D) and the serum mannose binding protein (MBP) [13]. These molecules have a collagen tail and a carboxy-terminal lectin domain. The collagen tail is a ligand for the collectin receptor, which is found on a variety of mammalian cells including monocytes, endothelial cells and fibroblasts. The lectin domain recognizes carbohydrate (CHO) moieties on the surface of viruses, bacteria, fungi, and protozoa. The other member of the C-type lectin family is the mannose receptor and is found on antigen presenting cells (i.e. macrophages and dendritic cells) [15]. This cell-surface protein directly binds CHO moieties found on the surface of pathogens, leading to phagocytosis of microorganisms [16-17].

The recognition of CHO moieties by members of the C-type lectin superfamily is mediated by structurally related calcium-dependent CHO-recognition domains (C-type CRDs) [13]. These domains recognize the equatorial orientation of the C_3 and C_4 hydroxyl groups in hexoses, N-acetylglucosamine, glucose, fucose and mannose [14]. These carbohydrate structures are common components of cell membrane and cell wall structures in a variety of pathogens, and include molecules like LPS, LTA, and mannans. In addition, the specific arrangement of CRDs on the host cell surface favor the spatial orientation of the CHO moieties that decorate the cell walls of microorganisms, providing they span the correct distance between the CRDs (ligands have to span the distance of 45 angstroms) [14, 18]. These conditions mediate high affinity binding of ligand to the receptor. The configuration of the hydroxyl groups in the sugars that decorate mammalian glycoproteins (i.e. galactose and sialic acid), and the repetitive CHO nature of many microbial PAMPs, are not accommodated by the CRD of C-type lectins. Thus, the broad selectivity of the monosaccharide-binding site, combined with the geometry of multiple CRDs in the intact lectins, allows these PRRs to mediate discrimination between self and non-self.

1.5.2 Mannose Binding Protein (MBP)

MBP is an acute phase serum protein synthesized by the liver that acts as both opsonin and activator of the complement cascade [19-20]. MBP has a relatively non-selective pattern of recognition, having the capacity to bind to the surface of a variety of infectious agents including viruses, gram positive and gram-negative bacteria, yeasts, and protozoan parasites [15]. In the case of protozoan pathogens, MBP specifically recognizes glucans, lipophosphoglycans and glycoinositolphospholipids that are composed of mannose, glucose, fucose or N-acetylglucosamine as terminal hexoses [21-22]. MBP is very efficient in recognizing microbial surfaces with a high content of repetitive mannose and/or N-acetylglucosamine residues, such as those found on *Candida albicans*, *Escherichia coli*, *Salmonella typhimurium*, *Neisseria gonorrhoeae*, *Leishmania spp*., and the human immunodeficiency virus (HIV) [15, 23-24]. Binding of CHO structures on the surface of these microorganisms often results in direct killing of the pathogens via activation of the complement pathway and subsequent formation of the lytic membrane attack complex (MAC). MBP can substitute for C1q in the activation of both classical and alternative complement cascades [19-20]. This protein is usually associated with small enzymes known as MBP-associated serine proteases (MASPs) [25-26]. Engagement of ligands by MBP results in the activation of the MASPs, which in turn activate the C3 convertase of the complement cascade that can also lead to opsonization of the pathogen by various complement components. Opsonization promotes phagocytosis of the pathogen by macrophages [23]. In addition, MBP can directly promote macrophage phagocytosis. Patients with deficiencies of MBP often suffer from recurrent bacterial and fungal infections, implicating the importance of this lectin in innate immunity, particularily during the early stages of microbial infection [27-30].

1.5.3 Mannose Receptor

The mannose receptor is a 175 kDa type 1 membrane glycoprotein that is expressed in high levels on tissue macrophages [31-32]. The receptor contains five domains; 1) an amino-terminal, extracellular cysteine-rich region, 2) a fibronectin type-II repeat-containing domain, 3) eight tandem lectin-like CHO domains, 4) a transmembrane domain, and 5) an intracellular carboxy-terminal tail [16, 33-34]. Like MBP, the mannose receptor binds sugars such as L-fucose, D-mannose, D-N-acetylglucosamine and D-galactose that are not common in terminal positions on mammalian oligosaccharides, but are frequently found on the surfaces of microorganisms [35] [36]. The mannose receptor can mediate the internalization of both soluble and particulate antigens (by endocytosis and phagocytosis, respectively) and is a prototypical PRR capable of binding a wide range of ligands found on the surface of pathogenic organisms [31]. Mannose receptors can recognize a number of pulmonary pathogens and has been implicated in the uptake of *Candida albicans, Escherichia coli, Pseudomonas aeruginosa, Pneumocystis carinii, and Mycobacterium tuberculosis* [17, 37-42]. This receptor also recognizes the protozoan parasites *Trypanosoma cruzi*, *Plasmodium yoeli*, *Leishmania donovani*, and *Leishmania mexicana* [43-45].

The tissue distribution of this receptor suggests a primary role for the clearance of soluble and particulate antigens. Phagocytosis mediated by the MR can induce cytocidal mechanisms and proinflammatory cytokine production [37, 46-47]. Functionally, ligation of the receptor results in release of lysosomal enzymes [48], and production of reactive oxygen intermediates (ROI) [17, 49], arachidonic acid metabolites [50], and cytokines [51-53]. Up-regulation of the Fc receptor and alterations in intracellular calcium fluxes and tyrosine kinase activation have also been implicated following ligation of the mannose receptor [51, 54-56]. The downstream effects of mannose receptor ligation are variant and are likely dependent on the differentiation or activation stage of the macrophage, the type of ligand, and cooperative actions of the mannose receptor with other macrophage lectins.

1.5.4 Complement Receptor (CR3)

The complement system is a proteolytic cascade that is activated either directly or indirectly by non-self recognition (i.e. microorganisms) and plays a major role in mammalian innate immunity [57]. There are three generalized pathways leading to activation of the complement cascade. The classical pathway requires antibody and the first complement components. The alternative pathway is directly activated by microorganisms and the lectin pathway requires MBP and associated MASPs (described above) [21, 25-26]. Activation of complement by microorganisms can have three major biological effects: 1) fixation of the terminal complement components resulting in complement-mediated lysis through the formation of a membrane attack complex (MAC); 2) fixation of the third complement component (C3) leading to opsonization and phagocytosis by macrophages; and 3) elaboration of the complement anaphylotoxins C3a and C5a leading to recruitment of immune cells and initiation of an inflammatory reaction.

Macrophages contain complement receptors on their surface that aid in the receptor-mediated phagocytosis of opsonized microorganisms [58]. The CR3 belongs to the CD18 family of integrins, which includes p150, 95, LFA-1 and CR3 [59-61]. Macrophage CR3 expresses two distinct binding sites; 1) a peptide binding site which binds such proteins as the complement component C3bi [62], and 2) a LPS-binding site that has also been shown to directly bind lipophosphoglycan (LPG) present on the surface of protozoan parasites like *Leishmania spp* [63-65]. Many pathogens are recognized by CR3 and this receptor has been implicated in the internalization of many different pathogens including, the yeast stage of *Histoplasma capsulatum* which uses the CR3/LFA-1/p150, 95 family of adhesion molecules to enter human macrophages using an opsonin-independent mechanism [66]. *Legionella pneumophila* uses CR1 and CR3 following opsonization with C3b and C3bi, respectively [67]. *Mycobacterium leprae* and both the promastigote and amastigote stages of *Leishmania* spp require C3 opsonization for uptake by CR3 [68]. Some pathogens such as *Leishmania* spp can even exploit the fixation of complement onto their surfaces in order to increase their uptake into host macrophages through the CR3 receptor [69].

1.5.5 Scavanger Receptors (SRs)

Scavanger receptors are a unique type of receptor that bind to both host- and pathogen-derived ligands, using pattern-recognition that does not induce macrophage activation [70-71]. These receptors were originally identified by their ability to bind modified low-density lipoproteins (LDLs), such as oxidized LDL (OxLDL) and acetylated LDL (AcLDL), and initially studied for their role in angiogenesis and LDL-cholesterol accumulation by macrophages in atherosclerotic lesions [72-73].

Other studies demonstrated that SRs also participate in evolutionarily conserved immune-related processes such as recognition and internalization of pathogens [73-77], cellular adhesion [73, 78-79], recognition of and engulfment of apoptotic cells [80], clearance of bacterial products from the circulation (i.e. LPS and LTA) [76-77], and wound healing [81-82]. Wounding exposes molecular structures and patterns on damaged cells or tissues that are not found on normal cells [74-83]. The types of pattern recognition used for identifying infectious microbes may also mediate wound recognition and monocyte adhesion through SRs.

Two classes, SR-AI and SR-AII, were the first macrophage SRs to be identified and were generated from alternative splicing of mRNA transcribed from the same gene [73, 85-86]. SR-A is found on monocytes, B-lymphocytes, capillary endothelial cells, platelets, and adipocytes. Both classes exhibit nearly identical ligand-binding properties, specifically binding an array of polyionic ligands with high affinity [73, 76-77]. SR-A recognizes polyionic molecules via its collagen-like domains, and recognition may be determined by the spatial characteristics of the repeating charged units found on host-derived, synthetic, and microbial origin. For example SR-A binds to apoptotic thymocytes [80], dextran sulphate [73], and intact gram positive bacteria and LTA found on various microorganisms, including *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Staphylococcus aureus*, *Enterococcu hirae*, and *Listeria monocytogenes* [76-77]. A role for SRs for the protection of mice from gram negative sepsis has also been reported [76]. While SRs can bind and internalize many different ligands, they do not appear to play a role in the activation of immune cells [70]. Therefore, SRs may participate in host defense by clearing foreign products such as LTA, LPS, or intact bacteria from tissues and the circulatory system during bacterial sepsis.

A third class of SRs, termed MARCO or SR-BI, has been identified and shown to bind both AcLDL and bacteria [87-88]. Class B SRs are composed of members of the CD36 family [89] and include SR-BI, the lysosomal protein Limp II [90], and *Drosophila* emp (epithelial membrane protein) [91]. This class of SRs binds a variety of ligands that must contain negatively-charged moieties in order to be recognized [92]. SR-BIs (i.e. MARCO), are primarily lipid-binding proteins that are expressed by cells and tissues involved in host defense and/or lipid metabolism [93] [94-95]. LPS associated with high-density lipoproteins and anionic environmental particulates appear to bind to SR-BI. Therefore, this class of SRc may facilitate LPS-clearance by the liver and the removal of particulates by alveolar macrophages in the lung [96].

1.5.6 CD14

The recognition of bacterial endotoxin (i.e. LPS) is an important function of the innate immune system, and failure to contain bacterial infections can result in septic shock as a result of the release of LPS from bacteria as they grow, multiply, or die (i.e. after antibiotic treatment). Therefore, eukaryotes have developed sensitive immunological surveillance mechanisms that can detect minute amounts of bacterial LPS [97-100]. The PRR responsible for recognition of this bacterial PAMP is CD14 (reviewed in [101]). CD14 is a 356 amino acid leucine-rich glycoprotein [102] that is expressed both in the plasma as soluble CD14 (sCD14) and as a glycosylphosphatidylinositol (GPI)-anchored protein on leukocytes (mCD14) [103]. A serum LPS-binding protein (LBP) also plays a role in LPS recognition and is often the first to bind to LPS. This LBP acts as a plasma lipid transfer protein that moves LPS monomers from bacterial membranes to a binding site of CD14 [104-105]. In combination with the serum LBP, CD14 allows effector cells (i.e. macrophages) to be triggered by sub-picomolar concentrations of LPS and both CD14 and LBP recognize the biologically active moiety of LPS (i.e. lipid A) [98]. Transfection of mCD14 cDNA into cell types that normally lack this surface receptor confers sensitivity to LPS [106-108]. Conversely, LPS-hyporesponsiveness is exhibit by macrophages defective in CD14 expression [109], confirming that the expression of mCD14 confers LPS responsiveness [97].

Two forms of the soluble LPS receptor (CD14) are constitutively generated; a 55 kDa form is liberated by escape from GPI-anchoring, and a 49 kDa form that is derived from the cell membrane by proteolytic cleavage with a serine protease [103]. sCD14 mediates the binding of LPS to endothelial cells that do not contain a surface LPS receptor (mCD14) [110-112]. This has been shown to potentiate LPS-responsiveness in endothelial cells resulting in the expression of adhesion molecules [110-112], and cytokines [112]. Alternatively, trimeric complex of LPS-sCD14-LBP can bind to nonmyloid cells without inducing cellular activation. Since this complex is internalized, this has been interpreted as an LPS-scavenging mechanism possibly for the removal of large amounts of endotoxin at infection sites [113]. Binding of LPS-sCD14 or LPS-LBP complexes to mCD14-positive cells followed by internalization can also be dissociated from signaling and may serve as another mechanism for LPS clearance [7].

Activation of macrophages following recognition of bacterial LPS leads to the production of tumor necrosis factor-alpha (TNFα) [97] and other pro-inflammatory cytokines including IL-1, IL-6, IL-8, and tissue factor [114-115]. The uncontrolled release of these cytokines results in many of the symptoms associated with septic shock including systemic activation of macrophages, increased vascular permeability, edema, and multi-organ failure. In polymorphonuclear (PMN) cells, mCD14 participates in the integrin-dependent adhesion to fibrinogen, complement-independent phagocytosis of gram-negative bacteria and phagocytosis of apoptotic cells [7].

Interestingly, the GPI-anchored mCD14 is unable to transduce a signal in response to ligation of LPS and requires an accessory signal in order to initiate a response (i.e. increased gene transcription). The mCD14 found on macrophages is co-expressed and forms a complex with another receptor known as the Toll-like receptor-4 (TLR4). The association of mCD14 with TLR4 has recently provided investigators with a link between LPS-binding and the transcriptional responses of macrophages to this bacterial PAMP.

1.5.7 Toll-like receptors (TLRs)

TLRs have received considerable attention as innate PRRs that are important not only in the recognition of PAMPs but are important in the initiation and transduction of the intracellular signals that induce innate immune mechanisms in macrophages [6]. The Toll receptor was originally identified as a key mediator of development in *Drosophila* [116], and only later was it found to also initiate innate antifungal responses in the fly [9]. Toll-receptors are characterized by an N-terminal extracellular domain containing several leucine-rich repeats (LRRs), and an intracellular C-terminal Toll/Interleukin-1 receptor (TIR) domain, named for its homology to the signaling domain of the IL-1 receptor. The TIR domain interacts with a heterotrimeric complex of death-domain containing adapter proteins [117], ultimately leading to the activation of the transcription factor NF-κβ or its homologues, Dif or Relish, in *Drosophila*.

Several TLRs have been identified in mammals and represent a unique class of PRR, with each TLR recognizing different PAMPS or combinations thereof (reviewed by [118]). Activation of TLRs by microbial ligands activates intracellular signaling events that lead to activation of NF-κβ and the MAPK cascades (c-Jun N-terminal kinase, p38, and ERK) [119-127]. The resultant effect induces innate immune mechanisms such as the production of ROI and RNI, chemokine/cytokine secretion, and cellular differentiation, many of which are regulated by the NF-κβ pathway [6].

The ligand specificities of different TLRs are extremely diverse [118]. For example, TLR-4 has been identified as the signal transduction component for bacterial LPS in humans [128-130]. TLR-4 associates with CD14 and a secreted linker protein known as MD2, to form the functional signal transduction receptor for LPS [131-135]. MD-2 also associates with TLR-2 to mediate signaling in response to lipopolypeptides from Gram-positive bacteria [136-138]. TLR-2 recognizes many different microbial components such as the glycosylphosphatidylinositol (GPI) anchors of parasitic protozoa (*Plasmodium falciparum* and *Trypanosoma cruzi*) [139-141], peptidoglycan from Gram-positive bacteria, lipoproteins and lipopeptides, lipoarabinomannan from *Mycobacterium tuberculosis*, and the yeast cell wall component zymosan [142]. Bacterial flagellin, is believed to be the ligand for TLR-5, and has been shown to induce a potent NO response in cultured epithelial cells via degradation of the inhibitory complex of NF-κβ (Iκβ) that regulates NF-κβ activity [136, 143-144]. Bacterial CpG DNA acts as the ligand for TLR-9 and has also been shown to induce NO production in macrophages [145]. Double stranded RNA is the ligand for TLR-3 [146] and imidazoquinolines (i.e. anti viral compounds) and guanine nucleoside analogs are recognized by TLR-7 [147-148]. The wide variety of ligands recognized by different TLRs and subsequent intracellular signaling pathways initiated in response to these ligands may also involve the formation of homodimers and even heterodimers after different TLRs interact with their ligands. For example, TLR-2 appears to recognize the largest variety of ligands, which is apparently due to the heterodimerization needed for TLR-2 mediated responses [142].

In addition to the recognition of foreign material, there is increasing evidence that TLRs can also recognize endogenous proteins, which leads to the induction of immune responses. In *Drosophilia*, an extracellular serine protease cascade is initiated in response to fungal and Gram-negative bacterial infections [149]. This cascade is responsible for cleaving a host protein, Spatzle, which is believed to function as the extracellular ligand of the *Drosophila* Toll receptor [9]. In mammals, it is becoming increasingly apparent that TLRs have an important role in the recognition of endogenous proteins, such as necrotic cells, and extracellular breakdown products (reviewed by [150, 151]). A variety of endogenous ligands have since been implicated as potential activators of TLRs and include fibrinogen [10], fibronectin [11], surfactant protein-A [152], heat-shock proteins [153-154], hyaluronan [155], and heparan sulfate [156]. Interestingly, the activation of mammalian TLRs by endogenous ligands may also require initiation of a serine protease cascade [157].

Until recently there was no conlusive evidence that TLRs existed in fish, as there were no published reports on the identification of an expressed teleost TLR homologue. Others have suggested that teleost TLRs may be present and important for the generation of immune responses in fish. For example, several EST projects have identified sequence fragments with significant similarity to regions of mammalian TLRs (Zebrafish: GenBanks accessions BM185313, BG304206, BF158452; Japanese flounder AB076709, AU091257; Rainbow trout: AF281346); however, none of these sequences contain both the TIR and LRR domains, which are hallmarks of the TLR family. The recently released *F. ruburipes* genome database [158], and analysis for fish homogues of the TLR family revealed that the Toll family is shared by fish and humans. The predicted Fugu TLR-2, -3, -5, -7, -8, and –9 corresponded structurally to the respective mammalian TLRs and one Fugu TLR showed equal amino acid similarities to human TLR-1, -6, and –10 [159]. Interestingly, two of the Fugu genes were found to be unique to fish and were named TLR-21 and –22. The pufferfish genome provides evidence of several possible TLR genes, and we have recently verified that at least one of these receptors is expressed on goldfish macrophages [160]. This goldfish TLR does not have distinguishable sequence homology with any single mammalian TLR that has been described but contains both a LRR and a TIR domain. Further studies are required to elucidate the specific functional characteristics of this goldfish macrophage TLR.

1.6 Antimicrobial mechanisms of macrophages

Macrophages function as both modulators and effectors of immunity. However, the primary and most fundamental role of macrophages in host defense is to limit the initial dissemination and/or growth of infectious organisms. Consequently, non-self recognition mechanisms are intricately linked to the process of phagocytosis, and are critical in the induction of macrophage-mediated immune mechanisms.

Macrophages possess a repertoire of potent pre-formed antimicrobial molecules stored within their granules and lysosomes. These organelles contain a salvo of degradative enzymes and antimicrobial peptides that are released into the phagolysosome upon ingestion of a foreign organism. In most cases, degradative enzymes such as proteases, nucleases, phosphatases, esterases, lipases, and highly basic antimicrobial peptides actively destroy the phagocytosed organism [161-166].

Although lysosomal-mediated degradation is a relatively efficient barrier to infection and colonization by potential microorganisms, several pathogens *parasitize* macrophages, utilizing them as a host cell for growth, replication, and/or maintenance of their life cycles. These include protozoan parasites such as *Toxoplasma gondii*, *Trypanosoma cruzi*, and *Leishmania spp*. This immunological paradox is difficult to comprehend: why would some parasites 'choose' to live within the very cells destined to destroy them? This paradox is easier to rationalize if viewed from the parasite's perspective. Since macrophages are often the first cells encountered by infectious organisms, their ultimate survival depends on their ability to prevent or circumvent lysosomal destruction by macrophages. Thus, many infections in vertebrates, and the diseases they cause, are a direct result of the adaptations of these infectious agents to survive within macrophages and to evade conventional phagocytosis-related destruction.

However, macrophages may be subsequently triggered to actively destroy persistent phagocytosed microorganisms. Recognition of microbial molecules in conjunction with cytokine stimulation results in the induction of an array of macrophage cytotoxic effector mechanisms. This cytokine-dependent set of reactions are collectively known as "macrophage activation", the process of which transforms a normally quiescent macrophage into an efficient killer cell, capable of producing a number of highly toxic molecules and inducing deprivational mechanisms that starve microbial pathogens of essential nutrients. Induction of macrophage activation is a highly regulated process, preventing unnecessary tissue destruction by activated macrophages at the site of inflammation.

This portion of the review provides a brief overview of the microbicidal strategies employed by activated macrophages to limit growth and/or replication of parasitic protozoans. Recent findings indicate that many of these macrophage antimicrobial mechanisms, and their regulation by cytokines, are not exclusive to higher vertebrates (i.e. mammals) but also exist in lower vertebrates such as fish. The following section highlights structurally and functionally the role of these macrophage-mediated killing mechanisms in host defense against infectious agents in mammals and fish.

1.6.1 Nutritent deprivation mechanisms

1.6.1.1 Recruitment and mobilization of iron-binding proteins

Commonly associated with infection and/or neoplasia is a condition known as anemia of infection and chronic disorder [167-168]. This condition was originally thought to be a pathological state induced by infection, but is now known to be a physiological response to infection, the desired effect of which is to decrease circulating iron, preventing or limiting the access of this critical metabolic element to pathogens [169-170]. Experimental evidence supporting the concept of induced iron deprivation as an antimicrobial mechanism has been summarized by Weinberg [169]: 1) hosts mobilize iron-binding proteins at sites of infection; 2) hosts recruit iron withholding mechanisms in response to microbial infection; 3) increased iron withholding, decreases incidence and intensity of infection; and 4) pathogenic microorganisms attempt to acquire iron from host tissues and fluids.

Iron sequestered in macrophages represents a significant portion of the total metabolically available iron content in mammals [171]. Iron transported systemically by serum transferrin, enters cells through CD71 receptor-mediated endocytosis, and dissociates from its receptor within the acidified endolysosome, and is subsequently transported inside the cell via a transporter system [172]. Internalized iron accumulates in labile iron pools and is compounded to low molecular weight proteins [171]. This intracellular pool acts as a readily available source of iron for metabolic processes or use by pathogens [171-173]. Excess iron in the cell is transferred to ferritin for storage [171]. Ferritin is one of many iron-containing proteins that are susceptible to inhibition by NO, which may represent a way of limiting the availability of intracellular iron to a developing pathogen [174]. Expression of ferritin is downregulated in activated macrophages, possibly through the effects of nitric oxide on iron response factors. These iron response factors are NO sensitive enzymes whose function is to regulate the status of iron in the cell [175-178].

Lactoferrin, another iron-binding protein found within the granules of neutrophils, has extremely high affinity for iron at low pH [179]. Lactoferrin participates in killing of pathogens by: 1) binding iron at localized sites of infection [173]; and 2) catalyzing the formation of hydroxyl radicals from superoxide anion produced by the respiratory burst [180]. When released by neutrophils, lactoferrin is scavanged by activated macrophages, augmenting their antimicrobial response [173].

Transferrin is a serum protein primarily involved in the transport of iron throughout the body and contributes to the deprivation of this essential element. When activated with cytokines, murine macrophages increase production of transferrin [181]. Transferrin produced by activated macrophages binds to intracellular iron, which limits the availability of intracellular iron for certain intracellular organisms [173]. Furthermore, activated macrophages have decreased numbers of transferrin receptors on their surface, limiting the influx of extracellular iron [182-185]. Interestingly, blood monocytes do not possess transferrin receptors on their surface, but acquire these receptors upon maturation or in response to inflammatory signals [184-186].

Infectious organisms require iron for growth and survival, and their growth, in most cases, is enhanced when excess iron is available [168]. However, excess iron can be detrimental to some pathogens, especially if they are susceptible to hydroxyl radicals, formed by superoxide anion and hydrogen peroxide in the presence of iron containing enzymes [49,187].

Iron metabolism and immune activation of macrophages are intimately linked to other antimicrobial mechanisms such as NO production and tryptophan degradation (see below). Treatment of murine macrophage with iron (Fe^{3+}) reduces activity of iNOS and chelation of iron, by addition of desferrioxamine, increases activity of iNOS [176, 188] and induces increased tryptophan degradation in human macrophages [170, 189]. Treatment of murine macrophages with IFNγ and LPS activates iron regulatory proteins (IRP), through NO-induced activation of IRP, subsequently leading to translational repression of ferritin synthesis and iron storage 304 [174]. Conversely, cytokines such IL-4 and IL-13 inhibit the actions of IFNγ and LPS on ferritin synthesis, mediating increased production of ferritin and expression of transferrin receptor expression, thereby promoting the uptake and utilization of intracellular iron [178].

1.6.1.2 Natural-resistance-associated macrophage proteins (NRAMP)

NRAMPs are members of the *solute carrier* family of proteins, and act as divalent metal/proton co- transporter proteins [190]. These proteins are highly conserved across an extremely diverse range of taxa, and homologues of this protein have been characterized in mammals, birds, fish, insects, yeast and bacteria [190-191]. In mammals, NRAMP was originally characterized as the protein product of a genetic resistance marker called the *Ity/Lsh/Bcg* locus, a genetic locus controlling innate macrophage-mediated immunity to a variety of unrelated microorganisms, including species of the genus *Salmonella*, *Mycobacterium* and *Leishmania* [192-196].

Two forms of NRAMP exist in mammals, NRAMP-1 and NRAMP-2, and share approximately 63% amino acid sequence homology [197]. NRAMP-1 is 110 kD integral membrane phosphoglycoprotein, primarily expressed in macrophages and granulocytes [198]. A single nucleotide substitution in Gly^{169} to Asp^{169} is responsible for a dichotomy in mouse strain susceptibility to intracellular pathogens such as *Leishmania major*, with genes bearing these mutations leading to a rapid degradation of the protein and lack of expression of NRAMP-1 in macrophages from susceptible strains of mice [198-200]. NRAMP-1 appears to be exclusively expressed in the lysosomal membrane of phagocytic cells [201], and message expression of the NRAMP-1 gene is induced in response to IFNγ and LPS [202].

NRAMP-1 is localized to late endosome/lysosomal intracellular compartments in macrophages and is recruited to the phagosome during its maturation to a phagolysosome [203]. It is generally believed that NRAMP-1 acts as an efflux pump, removing divalent cations from the lumen of the phagolysosome and transporting it into the cytoplasm [190, 204-206]. The co-transport activity of NRAMP-1 also leads to increased H$^+$ concentrations in the phagolysosome, aiding in the acidification process of the intra-phagolysosomal milieu and subsequently leading to activation of a number of lysosomal degradative enzymes [200].

Message expression for NRAMP-2 is regulated by alternative RNA splicing mechanisms that produce two different transcripts, one that contains an iron response element (isoform I) and another that does not (isoform II) [207]. NRAMP-2 is localized predominantly to the plasma membrane, and mediates iron uptake from acidified endosomes formed during transferrin receptor-mediated endocystosis [201]. Iron transported by NRAMP-2 subsequently associates with the cytoplasmic labile iron pool [208]. Other divalent cations transported by NRAMP-2 include, Zn^{2+}, Cd^{2+}, Mn^{2+}, Cu^{2+}, and Co^{2+} [190].

Treatment of macrophages with bacterial LPS induces a seven-fold increase in message expression for NRAMP-2 [209]. The sensitivity in expression of NRAMP-2 to stimulation with non-self molecules suggests a role of NRAMP-2 in host defense, even though NRAMP-2 has a ubiquitous tissue distribution [197]. Its potential role in macrophage-mediated innate immunity may be most influential during the initial stages of phagocytosis, in which divalent cations can be extruded from the phagosome prior to fusion of the phagosome with endosomes/lysosomes containing NRAMP-1.

The deprivation of iron and other divalent cations from the phagolysosome may induce cascading effects on pathogen survival. Divalent cations are co-factors for many enzymatic reactions, including those enzymes involved in oxidative phosphorylation, mitochondrial respiration, and DNA replication. Furthermore, many detoxifying enzymes produced by pathogens, such as superoxide dismutase, are dependent on divalent cations for functionality [190]. Limiting the availability of divalent cations may not only prevent growth and replication but also increase susceptibility of pathogens to the reactive intermediates generated by macrophages [190].

In fish, unlike mammals, it appears that the NRAMP2 gene is duplicated. In rainbow trout, two NRAMP proteins have been identified (designated as α and β) and both cluster with the mammalian NRAMP2. The expression of NRAMPα in rainbow trout was limited to the head kidney and ovary while expression of NRAMPβ was ubiquitous [210]. In puffer fish, two NRAMP2 proteins have also been found, one of them localizing to the late endosomes/lysosomes, consistent with a divergence towards an NRAMP1-like molecule [211]. Three NRAMP transcripts have been identified in catfish due to alternative splicing in the 3' UTR and alternative polyadenylation resulting in a single functional protein. Injection of catfish with LPS increased transcription of NRAMP2 in the kidney, spleen, and a monocyte/macrophage cell line [212-213]. In carp, NRAMP2 expression has also been observed and was found to be modulated by infections with *T. borreli* [214].

1.6.1.3 Tryptophan Degradation

Tryptophan is an essential amino acid, and is the least available amino acid for metabolism [215]. Plants and select microorganisms synthesise tryptophan *de novo*. Indoleamine 2,3-dioxygenase (IDO) is an inducible protein found in virtually all tissues of the body, and is involved in the catabolism of tryptophan [216]. This enzyme is a 42 kD protein that uses superoxide anion to oxidize the pyrrole ring of tryptophan in association with dihydroflavin mononucleotide and tetrahydrobiopterin as cofactors [217-218].

IDO induction in macrophages deprives intracellular pathogens of available tryptophan. Tryptophan degradation as an antimicrobial response was first suggested by Pfefferkorn [219], who demostrated that *T. gondii* required tryptophan for intracellular growth and survival in human fibroblasts. This inhibition of parasite growth was reversed by the addition of exogenous tryptophan to infected fibroblast cultures. The effect was not a result of decreased uptake of tryptophan, but due an enhanced degradation rate. Subsequent work by Byrne *et al.,* [220], also demonstrated the importance of tryptophan degradation in inhibiting the growth of *Chlamydia psittaci* in epithelial cells. The importance of tryptophan degradation in regulating the growth of these parasites and bacterial pathogens in macrophages has since been demonstrated [221-227]. In addition, metabolic products of tryptophan degradation, such as hydroxyanthranilic acid, have been shown to be toxic to microbes [216].

Indoleamine 2,3-dioxygenase is an interferon inducible protein [216], and cytokine-activated human macrophages have several fold higher IDO activity than do monocytes [221]. IFNγ is the most potent stimulator of IDO activity and tryptophan degradation [221, 228-230], and in some cases can induce an almost complete depletion of intracellular tryptophan stores [227]. Induction of IDO by IFNγ can be augmented by co-stimulating macrophages with TNFα [231]. IFNα and IFNβ are also inducers of IDO and tryptophan degradation, albeit to a much lesser extent than IFNγ [190, 229-231]. The addition of LPS to human monocytes stimulated with IFNα and IFNβ, enhances tryptophan degradation, comparable to that induced by IFNγ only [222, 230] suggesting that this antimicrobial response is also intimately linked to non-self recognition. The mechanism by which LPS enhances IFNβ degradation of tryptophan may involve an autocrine production of TNFα by macrophages. Treatment of monocytes with TNFα alone induces only minor amounts of IDO activity, whereas co-stimulation with IFNα and IFNβ causes enhanced tryptophan degradation [230].

Metabolic products of tryptophan degradation can induce other antimicrobial functions of activated macrophages. Picolinic acid, a metabolite of tryptophan degradation, augments NO responses in IFNγ–treated murine macrophages, and synergizes with IFNγ for induction of macrophage tumoricidal activity [232-235]. Picolinic acid exhibits its NO-inducing effects through a hypoxia-responsive element located 5' to the iNOS gene [236]. Picolinic acid also induces a rapid increase in production of the inflammatory chemokines MIP-1α and -β (macrophage inflammatory protein [237]) by macrophages; proteins involved in the recruitment of T cells to the site of inflammation [238-239].

1.6.2 The respiratory burst

1.6.2.1 Higher vertebrates

Phagocytosis is often accompanied by a dramatic increase in the consumption of oxygen by phagocytic cells. This *burst* in oxygen consumption is not solely required for the increased metabolic demands necessary for phagocytosis, since metabolic inhibitors such as cyanide do not significantly affect oxygen consumption by phagocytes [240]. A correlation between this oxidative burst and the formation of reactive oxygen intermediates has been clearly demonstrated [240-241].

The respiratory burst is a potent antimicrobial response of phagocytic cells such as macrophages and neutrophils [242-243]. The multi-component enzyme responsible for elicitation of the respiratory burst is known as the respiratory burst oxidase or the NADPH oxidase. This enzyme complex assembles itself on the inner surface of the plasma membrane upon appropriate stimulation [244].

The functional respiratory burst oxidase is comprised of at least 5 protein subunits. One constituent is a membrane cytochrome made up of two protein subunits, a glycoprotein of 91 kD (gp91*phox*), and a non-glycosylated protein of 22 kD (p22*phox*) [245-247]. Three cytosolic-derived proteins; a 40kD protein, a 47 kD phosphoprotein, and a 67 kD protein [248-251] form the rest of the functional oxidase. The cytosolic proteins exist as a complex in the cytosol [252-253], and after appropriate stimulation, translocate to the cytochrome component on the plasma membrane [254-257]. Translocation of cytosolic components to the cell membrane is mediated through the phosphorylation of multiple tyrosine and serine residues on p47phox and p67phox, due to the phosphorylation activities of a variety of kinases. As many as nine serine residues may be phosphorylated on p47*phox*, with enzyme translocation and activity dependent upon the phosphorylation of serine 303 and 304 [258]. The phosphorylation of cytosolic components mediates attachment to potential cytoskeletal elements, thus facilitating and directing translocation of the cytosolic components to the cell membrane [250, 259-260]. Upon attachment to membrane components, other kinases may phosphorylate distinct sites on the cytoplasmic-derived proteins [258, 261]. Kinases that are known to activate the respiratory burst oxidase include various protein kinase C isoforms [262-267], protein kinase A [265, 268-269], and mitogen activated protein kinases [265, 268]. Pharmacological inhibitors of specific MAPKs implicate both extracellular signal-related kinases (ERK-1 and -2) and p38 as important modulators of the respiratory burst [264, 270-272]. Cytosolic p47phox is directly phosphorylated by p38, ERK, and casein kinase II [264, 273]. However, casein kinase II phosphorylation of p47phox appears to mediate deactivation of the oxidase [273], demonstrating that phosphorylation can both activate and deactivate respiratory burst activity.

G-proteins are also involved in the regulation of respiratory burst function. Both Rac1 and Rac2 appear to be important in formation of the functional oxidase [274-280]. However, Rac-dependent activation of the respiratory burst is trigger-specific, since neutrophils isolated from Rac-2 deficient mice have normal respiratory burst activity when triggered with zymosan but display little or no respiratory burst activity when triggered with fMLP or PMA [281]. Rac proteins associate with an inhibitor G-protein known as Rho GDP-dissociation inhibitor, which prevents Rac from translocating to the membrane surface in resting cells [274, 282-283]. Another G-protein involved in regulation of oxidase activity is Rap1A, which associates with cytochrome components in the cell membrane and appears to function as a membrane targeting protein for the cytochrome [268].

NADPH is the substrate for the respiratory burst oxidase and binds to the cytosolic protein p67phox [284-287]. NADPH acts as a source of reducing potential for converting molecular oxygen to superoxide anion [286-289]. Production of superoxide anion (O_2^-) results in the spontaneous or enzyme catalyzed production of a barrage of reactive oxygen products including hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$), hypochlorous acid (OCl^-) and peroxynitrite ($ONOO^-$) [290-291]. Hydrogen peroxide is formed by the spontaneous mutation of superoxide anion, or through an enzyme catalyzed reaction involving superoxide dismutase. Hypochlorous acid is formed in the acidic phagolysosome through the reaction of chloride ions with hydrogen peroxide in the presence of the enzyme myeloperoxidase. Tissue macrophages can scavenge myeloperoxidase from exhausted neutrophils at the site of inflammation and use the enzyme for production of hypochlorous acid [292-294]. Hydroxyl radicals can be formed when hydrogen peroxide is oxidized by ferrous (Fe^{3+}) iron. Peroxynitrite is formed from the spontaneous reaction of superoxide with nitric oxide radicals ($NO^\cdot$) produced by the enzyme nitric oxide synthase.

Macrophages can be *primed* for enhanced respiratory burst activity by stimulation with various cytokines. Priming does not activate the NADPH oxidase, but enhances NADPH oxidase activity when macrophages are appropriately triggered (i.e. phagocytosis). Both interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα) are important cytokine mediators for priming respiratory burst activity in macrophages. IFNγ induces both an increased affinity of the NADPH oxidase for NADPH in macrophages [295-297] as well as an increased rate of gene transcription and message expression for the gp91phox protein [298-299]. IFN-γ does not affect p47phox transcription, indicating that p47phox availability may not limit the overall activity of the NADPH oxidase [298-299].

TNFα acts as an autocrine stimulus for enhancement of the respiratory burst function in macrophages [300], increasing expression of both p47phox and p67phox transcripts in macrophages [301]. Reactive oxygen intermediates produced during the respiratory burst response, in turn, augment production of TNFα by macrophages [302]. TNFα may also exhibit its priming activity via p38 MAPK-mediated exocytosis of granules containing the cytochrome component of the NADPH oxidase [302-303].

In many cases, the ability of cytokines to prime the respiratory burst response of macrophages depends on the sub-population of macrophage being studied. Monocytes are thought to exist in a semi-primed state *in vivo*, since freshly isolated monocytes triggered with PMA exhibit a vigorous respiratory burst response [295]. In contrast, monocytes cultured *in vitro* (monocyte-derived macrophages) have reduced respiratory burst activity, an effect that correlates with the length of *in vitro* cultivation [295]. *In vitro* stimulation of monocytes with IFNγ augments pathogen-triggered respiratory burst responses, but has no effect on priming of the respiratory burst activity of alveolar macrophages when triggered with the same stimuli [304]. The respiratory burst of bone marrow derived macrophages can be induced with zymosan but not PMA, while resident peritoneal macrophages show basal respiratory burst activity when triggered with PMA [305]. Bone marrow-derived macrophages are responsive to PMA as a trigger signal when cultured in the presence of GM-CSF, TNFα, IFNγ, or LPS [305].

Phagocytosis acts as a critical trigger for respiratory burst activity. Antibody (FcγR) and complement receptors are activators of the respiratory burst in macrophages and neutrophils. Cross-linking of FcγRI mediates activation of the immune-tyrosine activation motifs (ITAMs) via γ chain association, initiating the binding and activation of *syk*-family tyrosine kinases. *Syk* activation leads to subsequent downstream activation of phosphatidylinositol 3-kinase and phospholipase Cγ [306] that mediate the release of intracellular $Ca2^+$ and production of diacylglycerol. This event leads to activation of protein kinase C isoforms [307]. Phagocytosis via antibody or complement receptors requires protein kinase C activation [307-311]. Phagocytosis via antibody FcγRs concentrates PKC to the phagosome membrane, mediating localized activation of the respiratory burst oxidase [307]. Synthetic activators of protein kinase C, such as phorbol myristate acetate, are potent triggers of respiratory burst activity.

1.6.2.2 Lower vertebrates

The respiratory burst response has received considerable attention in teleosts. However, little is known about the biochemical structure of the enzymes involved in the respiratory burst of fish phagocytes. Secombes *et al.*, [312], demonstrated the presence of a low potential b-type cytochrome that localized to the plasma membrane of rainbow trout macrophages; a phenomenon similar to cytochrome b_{558} (gp91*phox* and p21*phox*) in mammalian phagocytes. A polyclonal antibody raised against a carboxy terminal sequence of human cytochrome b_{558} also recognized a 90 kD protein in eel neutrophils [313].

Significantly more work has been done regarding the regulation of respiratory burst activity in fish phagocytes stimulated by soluble mediators (i.e. cytokines). Since very few purified or recombinant fish cytokines have been available, early studies addressing the regulation of these responses in teleost macrophages have relied on the use of crude cytokine-like preparations. These crude cytokine preparations are obtained by stimulating kidney leukocytes with mitogens or macrophages with LPS and collecting the supernatants from stimulated cells. These crude cytokine preparations contain soluble mediators that have been shown to activate fish macrophages and neutrophils [314-326]. These preparations have also been shown to contain factors that deactivate antimicrobial responses of fish macrophages [319, 323, 327-328] indicating that the control of fish macrophage antimicrobial responses is mediated by a variety of endogenously-derived factors that exhibit 'cytokine-like' activities.

The respiratory burst of rainbow trout macrophages can be primed *in vitro* by stimulating resident kidney macrophages with culture supernatants obtained from mitogen stimulated kidney leukocytes [314, 315-316, 320, 329]. Priming the respiratory burst response of rainbow trout resident kidney macrophage requires extensive cultivation periods [i.e. 48 h] with these supernatants; an effect shared with their mammalian counterparts [330]. The macrophage activating factor (MAF) responsible for priming respiratory burst activity appears to be a product of fish T-cells [315], and is both heat and acid labile [316]. Production of a MAF that primes trout macrophage respiratory burst activity can also be induced by antigen-specific stimulation of lymphocyte cultures *in vitro* [331-332]. Crude MAF preparations also induce spreading and adherence of macrophages in culture [333].

Interestingly, different macrophage sub-populations in fish appear to display distinct priming kinetics of respiratory burst activity when stimulated with these crude-cytokine preparations. Our laboratory has developed a culture system for obtaining high yields of macrophages from the kidneys of goldfish [321, 334-336]. In this culture system, macrophages are generated by incubating kidney leukocytes in the presence of cell-conditioned medium (CCM) containing macrophage growth factor(s) (MGFs). We previously demonstrated that both kidney leukocytes and a goldfish macrophage cell line secrete an endogenous growth factor(s) that selectively induces the proliferation and differentiation of cells in the macrophage lineage. These *in vitro*-derived kidney macrophage (IVDKM) cultures appear to contain three distinct macrophage morphotypes, represented by macrophage progenitor cells, monocytes, and mature macrophages. Characterization of these different macrophage sub-populations was performedusing: 1) flow cytometric; 2) function (phagocytosis, respiratory burst, nitric oxide production); 3) cytochemical profiles (non-specific esterase, acid phosphatase, myeloperoxidase); 4) morphology; and 5) *in vitro* proliferation and differentiation pathways [321, 334-336].

The monocyte-like cells found in IVDKM cultures have a significantly greater basal respiratory burst response than do the more mature macrophage sub-population [337]. This is similar to what is observed in mammals where monocytes exist in a 'semi-primed' state and have a greater respiratory burst capacity compared to mature macrophages [295]. The monocytes present in goldfish IVDKM cultures can be rapidly primed for respiratory burst activity using crude MAF preparations, displaying enhanced respiratory burst responses after only 6-24 h of stimulation with crude MAF preparations [334-336]. Interestingly, after 24 h of stimulation with MAF, these cells gradually lose their primed respiratory burst potential [334-336].

The mature macrophage-like cells within IVDKM cultures display a different pattern of priming kinetics compared to the monocyte-like cells. The longer the macrophages are stimulated with MAF, the greater their respiratory burst response [334, 336]. These data are consistent with those observed by others using resident kidney macrophages isolated from various fish species [316, 329, 333, 338-340]. In these studies, respiratory burst capacity is most often measured ≥ 48 h of stimulation with MAF. Recently, differentiation-mediated alterations in antimicrobial functions have been observed in rainbow trout at the molecular level. MacKenzie *et al.,* reported that following stimulation with LPS, monocyte-like cells appeared to differentiate into more mature macrophage-like cells, which exhibited increased phagocytic capacities and expression of inflammatory genes [341].

The two functional sub-populations identified in IVDKM (i.e. monocytes and mature macrophages) display distinct priming kinetics that are similar to those described for mammalian phagocytes. In mammals, macrophages need extensive stimulation with IFNγ, (48-72 h) for induction of maximal respiratory burst activity [342]. Mammalian neutrophils, on the other hand, can be primed with IFNγ after only 6 h of stimulation, a consequence of protein upregulation and expression, and not an increased affinity change in the oxidase [343]. Thus, although the machinery required for respiratory burst activity may be similar in different cell types, functional regulation of this response may be specific to individual cells.

Although the native molecule(s) responsible for priming respiratory burst activity in fish have not been identified, we and others have attempted to purify these molecules from crude-cytokine preparations. Crude cytokine supernatants contain two distinct MAF activities that modulate macrophage respiratory burst activity [322] [334]. One activity (corresponding to a protein of 50 kD) induces a rapid but transient priming effect on the respiratory burst capacity of IVDKM. Stimulation of IVDKM with this molecule for only 6 h results in a greatly enhanced respiratory burst response compared to controls. However, 48 h after stimulation the respiratory burst capacity of stimulated macrophages is significantly reduced compared to those macrophages stimulated for only 6 h [319, 322, 334]. We have also demonstrated the presence of a 30 kD factor present in crude cytokine preparations that also modulates respiratory burst activity in goldfish macrophages. This molecule may be similar to one characterized by Graham and Secombes [316], who fractionated a respiratory burst enhancing molecule with similar molecular weight from rainbow trout. This molecule induces unique priming effects on IVDKM and cells stimulated with the 30 kD MAF continue to increase their priming potential the longer they are stimulated with this molecule [322, 334]. Interestingly, when IVDKM are co-stimulated with the 50 kD and 30 kD MAF, the respiratory burst potential of IVDKM is greater than that induced by either factor alone [322, 334]. However, the effect is transient, and IVDKM co-stimulated for 48 h with these factors have significantly lower respiratory burst potential compared to those stimulated for 24 h. The 50 kD MAF has also been shown to induce potent NO induction in goldfish macrophages [322, 334].

The respiratory burst of fish macrophages can also be primed by bacterial LPS [319, 344-347], β-glucans from yeast cell walls [348-352], and bacterial proteins [331]. Macrophage respiratory burst activity can also be primed *in vivo* by administration of killed or attenuated bacterial pathogens [329, 332, 339, 353] and neutrophils collected after injection of irritants such as casein or heat killed bacteria also display elevated respiratory burst responses [354]. Similar to the priming observed by stimulation with crude leukocyte supernatants, the priming of the respiratory burst by foreign agents (i.e. LPS) also requires extended cultivation periods.

There is also evidence to suggest that a TNFα-like molecule may also be responsible for the respiratory burst-inducing activity exhibited by crude cytokine supernatants [320, 326, 345, 355-356]. Studies have shown that human recombinant TNFα synergizes with crude-cytokine preparations to enhance priming of respiratory burst activity in rainbow trout macrophages [345, 352, 355]. Moreover, priming of the respiratory burst can be partially inhibited using anti-human TNFα receptor 1 monoclonal antibodies, suggesting a certain degree of conservation in both the TNF molecule and its receptor between mammals and teleosts [356]. Supernatants derived from rainbow trout were also highly toxic to murine L929 cells, which are highly sensitive to mammalian TNFα, further suggesting that a TNFα-like molecule does indeed exist in fish [326]. The presence of this cytokine has recently been confirmed by the cloning of the TNFα gene from a variety of fish species including rainbow trout, Japanese flounder, carp, and catfish [357-362]. Furthermore, the recombinant protein has been expressed and functional studies suggest that teleost TNFα plays a key role in the induction of inflammatory responses in fish [363] and may also be responsible for induction of ROI production in fish macrophages.

Another cytokine that plays an important role in the induction of macrophage antimicrobial responses in fish is IL-1 (reviewed by [364]). Recently, rainbow trout

IL-1 has been cloned [365-369], and subsequent studies have resulted in the cloning of the full-length gene in different fish species and also in shark [370-375]. This cytokine has been shown to prime the respiratory burst response in mammalian macrophages and neutrophils [305, 376-378]. Recently, recombinant trout IL-1 has been produced and functional studies performed [379-381]. The recombinant cytokine induced migration of head kidney leukocytes [379] and was recently shown to increase protection of rainbow trout from infections with *Aeromonas salmonicida*, a finding that correlated with systemic IL-1β, COX-2, and lysozyme II gene expression [381]. The protective effects of IL-1 may also result from the ability to induce production of ROI by fish macrophages as seen in mammals.

Another molecule that appears to affect respiratory burst function in teleost macrophages is growth hormone. Recombinant rainbow trout growth hormone has been shown to prime sea bass macrophage respiratory burst response after 24 h of stimulation [382-384]. Growth hormone administered *in vivo* also augments respiratory burst activity in rainbow trout neutrophils [385].

It has been shown that the respiratory burst response plays an important role in the destruction of several fish pathogens. *Renibacterium salmoninarum*, etiological agent of bacterial kidney disease, is susceptible to H_2O_2 killing by trout macrophages and addition of catalase to macrophage cultures inhibits killing of this fish pathogen *in vitro* [386]. Graham and Secombes [314] demonstrated that rainbow trout macrophages stimulated with crude MAF preparations could inhibit growth of the bacterium *Aeromonas salmonicida*. Subsequent work by this group, and others, demonstrated that killing of select pathogens correlated with the production of ROI, and the addition of scavengers of reactive oxygen, such as catalase, abolished the ability of macrophages to restrict the growth of pathogens [332, 345, 386].

1.6.3 Nitric oxide production (NO)

1.6.3.1 Higher vertebrates

It was demonstrated in the early 1980s that nitrogen oxides were common by-products of metabolism, and that the treatment of rats with bacterial endotoxin resulted in increased nitrate levels in body fluids [387-389]. Stuehr and Marletta [390] demonstrated that endotoxin-stimulated murine macrophages produce both nitrate and nitrite, and subsequent studies showed that the production of these NO by-products by macrophages correlated with an increased cytotoxicity against tumors and pathogens [391-396].

The enzyme responsible for production of NO is nitric oxide synthase [NOS] of which several isoforms have been identified, including nNOS (neuronal NOS), eNOS (endothelial-derived NOS), and iNOS (inducible NOS). The isoform expressed in activated immune cells is predominantly iNOS. iNOS requires the presence of Ca^{2+} and calmodulin for activity [397], but has a high affinity for both these factors at the low intracellular concentrations found within the cytoplasm, and therefore does not depend on transient Ca^{2+} influxes for activity [397-398]. Inducible NOS also requires tetrahydrobiopterin, flavin adenine dinucleotide and flavin mononucleotide for activity [397-403].

The guanidino nitrogen of L-arginine acts as the nitrogenous donor for the enzymatic reaction [392, 404-405], and analogs of this compound, such as N^G-monomethyl-L-arginine (N^GMMLA) and amino-guanidine, are potent inhibitors of this enzyme [406]. Increases in arginase transporter activity, argininosuccinate synthase and argininosuccinate lyase appear to be a prerequisite for iNOS production in macrophages [407-408]. Cationic amino acid transporters, such as CAT-2B, are also up-regulated in response to stimulation with LPS and/or IFN-γ, mediating the increased uptake of arginine during macrophage activation [409-410].

Free radical $NO^{\cdot}$ is thought to be the immediate enzymatic and microbicidal product produced by iNOS, although this still remains controversial [411-413]. Nitroxyl ion (NO-) may also be produced as the dominant reactive nitrogen species [411]. Secondary nitrogenous reactants such as nitrite, nitrate, and nitrosamines, produced as by products of the primary reactive species, are also toxic to pathogens [393, 406]. Although the respiratory burst has no apparent role in the production of NO [405], superoxide anion, produced from the respiratory burst response can react with nitric oxide to form peroxynitrite [$ONOO^-$], a reactive intermediate known to have potent microbicidal activity [414-415]. Furthermore, NOS enzymes also appear to have the capacity to generate reactive oxygen under specific circumstances, potentially facilitating the production of highly reactive peroxynitrite [416-417].

Nitric oxide has an extremely high affinity for iron and readily reacts with iron containing enzymes [418-419]. Enzymes susceptible to NO include the oxidoreductases of the mitochondrial electron transport chain, aconitase, protein kinase C, ferritin, indoleamine 2, 3- dioxygenase, and ribonucleotide reductase [174, 396, 418, 420-424]. These enzymes are involved in oxidative phosphorylation, mitochondrial respiration, intracellular signaling, iron storage, tryptophan degradation and DNA replication, respectively. Interestingly, low concentrations of NO may facilitate enhanced production of superoxide by activating the mitogen-activated protein kinases ERK-1 and –2 [425]. Higher concentrations of NO inhibit production of reactive oxygen intermediates [425]. In addition, iNOS itself is susceptible to NO, indicating that NO may function as a feedback inhibitor of its own production [422] [426-427]. The mechanisms for inhibition may be related to the ability of NO to stabilize IκBα activity, preventing translocation of NF-κβ to the nucleus [130] or by modulating the binding properties of NF-κβ to DNA target sequences [428].

Regulation of iNOS transcription and subsequent translation is complex, with various promoter and enhancer elements associated with the iNOS gene. Regulatory elements within the iNOS gene complex include hypoxic-responsive elements [236], NF-κβ binding sites [429-431], STAT binding sites [432-434], interferon regulatory factor binding elements [435-437], and an AP-1 binding transcription site [438]. Upstream cellular signaling elements, such as the MAP kinases ERK and p38 also appear to play a role in pathogen-mediated signaling of iNOS in macrophages as demonstrated by the inhibition of NO production by specific inhibitors of the ERK and p38 signaling cascade [439]. However, both ERK and p38 do not affect NF-κβ binding to target DNA sequences in the iNOS promoter [439], suggesting that their role in the induction of iNOS may be dependent on autocrine production of cytokines that affect NO [272, 439-440] or altering binding of other transcription factors to iNOS promoter sequences. Feng *et al.*, [439] demonstrated that inhibitors of p38 activity prevent binding of nuclear complexes to the interferon regulatory factor binding consensus sequence in the iNOS gene that correlate with reduced transcription of the iNOS gene.

Inducible NO cytotoxicity is an antimicrobial mechanism primarily attributed to activated macrophages, although neutrophils can also produce NO in response to immune challenge [441-444]. However, much of the work regarding the antimicrobial activities of NO has been carried out using murine macrophages. The production and regulation of NO by human macrophages has been controversial in the past [445] [446]. Early studies, speculated that NO may not be an antimicrobial mechanism of human macrophages; this based on the fact that the cytokines involved in induction of NO production in mouse macrophages did not induce human macrophages to produce NO [447-450]. However, several reports have demonstrated NO production and killing of pathogens by L-arginine dependent mechanisms in human macrophages [451-454], and the iNOS gene has been cloned from human macrophages [455-457]. It is generally accepted that iNOS is an antimicrobial response in human macrophages [445]. It is interesting to note, however, that production of NO by mammalian macrophages may be developmentally regulated, since human monocytes cultured *in vitro* over several days acquire the capacity to produce NO [451, 458-459]. This data suggests that monocytes require certain differentiation / developmental signals as pre-requisites for production of NO in response to stimulation with cytokines and foreign molecules. This hypothesis is further supported by the observation that murine monocytes recruited to the peritoneal cavity in response to inflammatory signals (after 48 h) display weak NO production, whereas those isolated after longer retention times in the peritoneal cavity (i.e., 12 days) have strong NO production [460]. This observation may explain the discrepancies observed between humans and mice, since monocytes are the predominant sub-population used in human studies and inflammatory or mature macrophages are often used in murine studies. Interestingly, this observation is also similar in fish, where monocytes have been shown to have a relatively weak NO response compared to more mature macrophage sub-populations [336].

1.6.3.2 Lower vertebrates

Inducible NO production by fish macrophages has been a recent discovery. Although constitutive nitric oxide synthase had been demonstrated from the central nervous system of fish [461-462] only one report prior to 1995 had demonstrated that fish possess an inducible form of this enzyme. Schoor and Plum [463], demonstrated inducible NO production, using enzyme histochemical techniques, from kidney homogenates obtained from channel catfish infected with the bacterium *Edwardsiella ictaluri*. Our laboratory subsequently demonstrated, that NO production could be induced in a goldfish macrophage cell line stimulated with bacterial LPS [464]. Crude cytokine supernatants were also shown to contain a factor(s) that synergize with bacterial LPS to induce goldfish macrophages to produce NO [318], an effect since demonstrated in several fish species [325, 332, 340]. Recently, nucleotide sequences for goldfish, rainbow trout, carp, zebrafish, and Atlantic salmon inducible nitric oxide synthase (iNOS) have been identified [465-472] and share approximately 60-70% homology with mammalian-derived macrophage iNOS [466]. Rainbow trout head kidney macrophages stimulated with 25-50 μg/ml LPS expressed maximal levels of iNOS between 2 and 6 h post-stimulation [466]. Furthermore, it was shown that the gills are an important site of iNOS expression in rainbow trout [467]. Following challenge with *Renibacterium salmoninarum* iNOS message was rapidly up-regulated in the gills (i.e. between 3 and 6 h) and lasted for several days. In comparison, a delayed expression of iNOS was observed in the kidneys of challenged trout (i.e. after 24 h) that was rapidly down-regulated [467]. Using a combination of biochemical, immunohistochemical, and immunoblotting analyses, iNOS immunoreactive cells in head kidney tissues of rainbow trout were identified as heterophilic granulocytes, and iNOS positive macrophages and neutrophils were found in the liver [468]. In carp, induction of the iNOS gene was dependent on NF-κβ and was observed following stimulation of carp phagocytes with LPS or *Trypanoplasma borreli*, which also correlated with the production of high levels of NO [470].

Unlike mammals where numerous studies have been conducted, relatively few studies have examined the NO-induced cytotoxic capability of fish macrophages *in vitro*. Recently, Yin *et al.*, [332] demonstrated that MAF activated catfish macrophages were bactericidal towards *Aeromonas hydrophila*, and that killing could be partially blocked using N^GMMLA, an inhibitor of NO production. Fish macrophages can be induced to produce NO in response to intracellular infection. For example, goldfish macrophages infected with *Leishmania major*, an obligate intracellular protozoan pathogen of mammalian macrophages, produce NO in the absence of any additional exogenous cytokine signals [323, 334]. Induction of this response appears to be mediated via the recognition of a foreign molecule by the macrophage, since phagocytosis of latex beads is insufficient for initiating NO production in goldfish macrophages [334]. This contrasts the scenario observed in mammalian macrophages, which require an accessory signal such as IFNγ for induction of the NO response [334]

[473-476]. Similar effects are also seen in fish macrophages infected with the Gram-positive bacterium *Mycobacterium chelonei* [338] or microsporidians (Neumann *et al.*, unpublished observations) as well as exposure to heat-killed *Trypanosoma danilewskyi* and *Aeromonas salmonicida* [477].

Recently, we characterized the molecules present in crude mitogen-stimulated goldfish kidney leukocyte supernatants that exhibited NO-inducing activity in goldfish macrophages. One of the important molecules capable of inducing NO response in fish macrophages is transferrin, and it appears to require enzymatic cleavage of the native protein [478].

1.7 Immunomodulatory roles of transferrin

Transferrin is a bi-lobed monomeric serum glycoprotein of approximately 70 to 80 kD and is responsible for the transport and delivery of iron to cells and is primarily produced in the liver [479-483]. The N- and C- terminal lobes of transferrin have similar amino acid sequence, tertiary structure and are believed to have evolved as a result of gene duplication [484-485]. The two homologues globular lobes contain deep clefts capable of binding iron and are connected by a small peptide region (~15 amino acids) called the inter domain bridge, which varies in length between different transferrin species [486-487]. Transferrin is abundant in nature and has been identified in a wide range of organisms (i.e. insects, crustaceans, fish, and mammals) [488-492]. There is also extensive structural and sequence homology between transferrins from different species [485].

Binding of iron to transferrin creates a bacteriostatic environment by limiting the availability of iron to replicating pathogens (Section 1.6.2.1). However, in addition to its primary described role as an iron-binding protein, transferrin appears to exhibit a variety of other biological functions. For example, transferrin induces neutrophilic end-stage maturation [493], supports the growth and differentiation of the human promyelocytic cell line, HL-60 [494], and selectively stimulates cellular proliferation of prostatic carcinoma cells [495]. Activation of casein kinase II, an enzyme involved in the regulation of cell growth, was shown to require the application of transferrin in combination with an insulin-like growth factor [496]. Transferrin up-regulates chemokine synthesis by human proximal tubular epithelial cells [497] and the addition of transferrin to rat cultured aortic smooth muscle cells induced a concentration- and dose-dependent increase in iNOS mRNA and nitrite accumulation [498]. Elevated transferrin concentrations in cerebral spinal fluid after subarachnoid hemorrhage also increased iNOS mRNA expression by smooth muscle cells [498]. A recent study demonstrated that in addition to the binding of iron, transferrin functions as a protein-binding protein [499] and is one of the constituents secreted by platelets that can activate phagocytosis [500].

In chickens, ovatransferrin is a key inducer of cellular activation measured by its ability to induce the production of IL-6 and matrix metallopreoteinases as well as the induction of respiratory burst in macrophages [501-502]. Ibranim *et al.*, have reported that ovatransferrin can directly contribute to the killing of bacteria [503]. This was demonstrated by the identification of a bactericidal domain in the amino-terminal half molecule (i.e. N-lobe, residues 1-332). The antibacterial properties of this domain were dependent on 3 intrachain disulfide bonds and the protein sequence within the N-lobe demonstrated a marked sequence homology to insect defensins that contained 6 highly conserved cysteine residues. Therefore, ovatransferrin is believed to be one of the key components found in inflammatory chicken serum that is capable of not only mediating immune cell functions but can also contribute to the direct killing of bacterial pathogens.

Teleost transferrin has also been described as an acute phase protein and increased levels of transferrin expression were observed following bacterial infections in rainbow trout [504]. In addition, we have shown that transferrin exhibits a novel function by inducing NO production in goldfish macrophages. [477-478]. Transferrin must be cleaved in order for it to activate fish macrophages to produce NO. The native protein (~55-60 kD) undergoes proteolytic cleavage in goldfish leukocyte cultures stimulated with mitogens and/or mixed lymphocyte reactions [478]. The resultant peptides of 33-37 kD synergized with LPS for induction of NO in goldfish macrophages. Products released from necrotic fish cells (i.e. macrophages and neutrophils) appear to play a major role in the cleavage of transferrin into its active NO-inducing form [477]. The connection between fish neutrophils, macrophages and transferrin provides an interesting model for understanding inflammation and regulation of the immune response of phagocytes in fish. During the initial phase of inflammation, vascular leakage of capillaries initiates swelling at the site of infection. In mammals, transferrin has been shown to leak into inflammatory sites during this early phase of inflammation [505-508]. In goldfish, serum components have also been found to leak into the peritoneal cavity following induction of an inflammatory response [509]. Neutrophils are one the first immune cells recruited to the site of inflammation, and their migration into the site may initiate the cleavage of transferrin via the production of neutrophil-derived proteases (i.e. elastase, gelatinase, matrix metalloproteases, etc.). We have recently shown that the intracellular contents of goldfish granulocytes were capable of cleaving transferrin [477]. Monocytes that are subsequently recruited to the inflammatory site, and resident tissues macrophages, may then recognize the cleaved transferrin products as a signal for initiating the production of NO. This would be analogous to the recognition of endogenous proteins by TLRs in mammals [150]. Although fish monocytes do not appear capable of producing NO [334, 336], transferrin may also initiate the differentiation of these cells into the more mature macrophage phenotypes, as is the case in mammals [185-186, 510-513]. This may cause monocytes to become responsive to signals initiating NO production. Differentiation of human monocytes into more mature phenotypes results in the acquired capacity to produce NO [451]. We have observed a similar effect in goldfish monocytes [334].

This novel finding implicates transferrin as primitive regulator of immune phagocyte function in lower vertebrates and possibly in higher vertebrates. Moreover, it is interesting to speculate that proteins homologous to transferrin may play an important role in the induction of the NO response in invertebrate immunocytes. It has been reported by several groups that invertebrate immunocytes possess the capacity to produce NO in response to immune challenge [514-515]. Since many invertebrates possess transferrin-like molecules [516], it is conceivable that proteolytic cleavage of transferrin-like molecules may represent a primitive form of immunoregulation of innate immunity, and specifically, macrophage antimicrobial functions. Furthermore, this process may be mediated by a highly conserved family of innate immune receptors such as TLRs, which we have recently described in goldfish [160].

1.8 Summary

The recognition and elimination of invading pathogens is vital for host survival. Macrophages play a central role in host protection and cells functionally reminiscent of the vertebrate macrophage are present in virtually all metazoan organisms, attesting to the importance of these phagocytic cells in host defense in all multi-cellular organism. Macrophages contain a repertoire of non-self recognition receptors (i.e. PRRs) that recognize molecular patterns found on pathogens surfaces called PAMPs, and many of these innate immune receptors are highly conserved throughout evolution (i.e. Toll and TLRs). Recognition of PAMPs by PRRs leads to the rapid phagocytosis of the invading microbe followed by their eventual destruction using a variety of preformed enzymes or production of reactive intermediates (i.e. ROI and RNI) by inducible antimicrobial pathways.

Phagocytosis is the ancestral defense mechanism of all metazoan animals and is essential in preventing the dissemination of infectious agents. Many of the antimicrobial effector responses of vertebrate phagocytes are similar across diverse animal taxa. Inducible antimicrobial responses such as the respiratory burst pathway and production of NO have been demonstrated in fish phagocytes, and display biochemical and physiological similarities to homologous responses induced in mammalian phagocytes. Both respiratory burst activity and NO induction have been shown to be critical effector mechanisms in limiting the growth of fish pathogens and studies addressing the regulation of these responses in fish have provided some novel insights into how these mechanisms are regulated in vertebrates.

CHAPTER 2

MATERIALS AND METHODS

2.1 Fish

Goldfish (*Carassius auratus*) and Carp (*Cyprinus carpio*) were purchased from either Ozark Fisheries Inc. (Southland, MI) or Grassy Forks Fisheries (Martinsville, IN). Rainbow trout (*Oncorhynchus mykiss*) were obtained from a local fish hatchery supplier. The animals were maintained at the Aquatic Facility of the Department of Biological Sciences, University of Alberta. Goldfish and carp were maintained at 20°C and rainbow trout at 15°C in a flow-through water system on a simulated natural photoperiod (Edmonton, Alberta). Fish were fed to satiation daily with trout pellets, and acclimated to this environment for at least three weeks prior to use.

2.2 Fish serum

Fish serum was obtained by bleeding large goldfish or common carp (15-40 cm). The fish were anaesthetized using tricaine methanosulfonate (approximately 40 mg l^{-1}) and bled by caudal venipuncture. These fish were maintained solely for serum production and were bled every 8-10 weeks. Blood collected from these fish was allowed to clot at room temperature for approximately 1 h and overnight at 4°C. The following morning blood samples were centrifuged at 1000 x *g* for 20 min and serum removed. Serum was heat inactivated at 56°C for 30 min, filter sterilized (0.22 μM), and stored at -20°C until used.

2.3 Fish cell culture

2.3.1 Culture medium

To make 2 liters of incomplete culture medium, the following chemicals were added to 700 ml of Milli-Q water; 7.0 g HEPES (Sigma), 0.688 g KH_2PO_4 (BDH), 0.570 g K_2HPO_4 (BDH), 0.75 g NaOH (Fisher), 0.34 g $NaHCO_3$ (BDH), 0.584 g L-glutamine (Sigma) and 0.01 g insulin (Sigma). The following solutions were then added, 1 L of a 50:50 (v/v) mixture of Leibovitz's-15 medium and Dulbecco's Modified Eagle Medium (Gibco), 80 ml of 10× HBSS, 25 ml each of MEM amino acid solution (50×), MEM non-essential amino acid solution (100×) and sodium pyruvate (100 mM) (Gibco), 20 ml MEM vitamin solution (100×; Gibco), 20 ml of nucleic acid precursor solution (containing 0.067 g adenosine, 0.061 g cytidine, 0.034 g hypoxanthene, 0.061 g thymidine and 0.061 g uridine /100 ml water) and 7 μl of 2-β-Mercaptoehtanol (Sigma). After the addition of all reagents, medium was balanced to pH 7.2 with 1 N NaOH and adjusted to a final volume of 2 L with Milli-Q water and filter-sterilized with a 0.22 μm filter (Millipore). Complete culture medium contained 100 μg/ml gentamicin, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% newborn calf serum (Hyclone), and 5% carp serum.

2.3.2 Isolation of fish kidney leukocytes

Fish were anesthetized with MS222 (Syndel) and killed by cervical dislocation. Kidneys were aseptically removed and placed into a petri dish containing ice-cold medium. Using a sterile plunger from a 3cc syringe, kidneys were gently pressed through sterile stainless steel screens to release kidney cells. Screens were rinsed with medium (12.5 ml/kidney) containing antibiotics (50 µg/ml gentamicin, 100 U/ml penicillin, 100 µg/ml streptomycin) and heparin (50 U/ml). The resulting cell suspension was layered on 51% Percoll (Pharmacia) and centrifuged at 400 x *g* for 25 min. Cells at the medium-51% Percoll interface were removed with a sterile pipette and transferred to sterile centrifuge tubes. To remove Percoll, cells were washed twice in serum-free medium and centrifuged at 200 x *g* for 10 min. at 4°C. Viable leukocytes were enumerated using a haemocytometer after staining with trypan blue (Gibco).

2.3.3 Generation of goldfish and carp primary macrophage cultures

Isolated kidney leukocytes (15-20 x 10^6 cells) were cultured in 20 ml of complete medium supplemented with 25% (v/v) cell conditioned medium (CCM). After 8 to 12 days of incubation at 20 °C, cells were harvested, enumerated and used as a source of macrophages for bioassays. Supernatants from these cultures were also collected and used as a source of CCM for establishing subsequent macrophage cultures.

2.3.4 Generation of trout-primary kidney monocyte cultures (T-PKM)

Rainbow trout kidney leukocytes were isolated by 51% Percoll were used for the generation of T-PKM cultures. The generation of T-PKM was performed using similar protocols for the establishment of goldfish and carp primary macrophage cultures. Briefly, trout kidney leukocytes (10 x 10^6 cells) were isolated using 51% Percoll, seeded into 25 cm^2 flasks, and incubated at 20°C. Microscopic and flow cytometric analysis was performed on alternate days (see below) during cultivation period. Initial cultivation trials were performed in the absence of CCM and supernatants from these cultures were collected to obtain trout-derived CCM, which was used for the establishment of subsequent cultures and generation of further CCMs [337]. Trout-derivd CCMs were routinely collected from 8-12 day old T-PKM cultures by centrifuging the cells at 200 x g for 10 min. The CCM was subsequently filter-sterilized (0.22 μm filter) and stored at 4°C. To test for CCM activity, leukocytes were seeded into 25 cm^2 flasks as described above and incubated in the absence (complete medium only) or the presence of CCM (25% v/v). T-PKM cultures were analyzed by flow cytometry after 4, 8, and 12 days of cultivation to assess the effect of CCM on development of trout cultures.

2.4 Flow cytometric analysis of fish macrophage sub-populations

Primary fish macrophage cultures were analyzed using a FACS Calibur flow cytometer equipped with a cell sorter (Becton Dickinson). Forward scatter (FSC; size) and side scatter (SSC; internal complexity) parameters were considered for examination of isolated fish leukocytes. All flow cytometry was performed on cells suspended in phosphate-buffered saline (1xPBS) or incomplete culture medium and 10 000 cells were routinely examined during analysis. The following flow cytometer settings were used for the analysis of goldfish/carp IVDKM and T-PKM, respectively; forward side scatter - photodiode set to E-1, E00, AmpGain set to 9.33, 1.05; and side scatter – photomultiplier voltage set to 455 V, 450 V, AmpGain set to 1.00, 1.00.

2.5 Mammalian cell lines

The mammalian macrophage-like cells (P388D.1) were used in select experiments for comparing macrophage responses between mammals and teleosts. The P388D.1 macrophage-like cell line, was maintained by serial passage in DMEM (Gibco) containing 10 % (v/v) fetal bovine serum and 50 µg/ml gentamicin.

2.6 Isolation and cultivation of bone marrow-derived murine macrophages

Bone marrow-derived macrophages (BMDM) were obtained by flushing the femurs of C57BL/6 mice with sterile 1XPBS. Cells were sedimented by centrifugation at 300 x g for 10 min and re-suspended at a concentration of 1×10^6 viable cells in DMEM with 10% FBS, 50 µg/ml gentamicin and 10% (v/v) L929 cell-conditioned medium (LCCM) (day 7 supernatants of cultured L-929 cells were used as the source of CSF-1). Bone marrow-derived cells were cultured in 50 ml sterile polypropylene centrifuge (Corning) and fed every 4 days with 1.5 ml of L cell-conditioned medium. The cells were used after 9 to 11 days of culture. To harvest, the cells were placed in ice for 30 min and centrifuged at 300 x g at 4°C for 10 min.

2.7 Production of mitogen-stimulated goldfish kidney leukocyte conditioned supernatants

Kidney leukocytes were isolated from 20 goldfish, pooled, enumerated, and seeded in 75 cm^2 tissue culture flasks at a concentration of 4×10^6 cells/ml. The leukocytes were then incubated overnight in medium containing 2.5% carp serum and 10% FCS at 20°C. The following morning (~18 h) the mixed leukocyte cultures were stimulated with 10 µg/ml Concanavalin A (Con A, Boehringer Mannheim), 10 ng/ml phorbol myristate acetate (PMA, Sigma), and 100 ng/ml calcium ionophore A23187 (Sigma). Cultures were incubated in the presence of mitogens for 6 h, after which the mitogens and serum were removed by washing the cells with three exchanges of 25 ml Hanks balanced salt solution (1xHBSS). The remaining cells were resuspended in incomplete culture medium and incubated for 72 h at 20°C. Every 24 h approximately 500 µl sub-samples were removed from the cultures, filter sterilized, and stored at 4°C prior to being analyzed by Western blot and/or the nitric oxide bioassay. After 72 h, the remaining supernatants from all flasks were pooled, filter sterilized, and stored at –20°C.

For some experiments, modifications to the preparation of mitogen-stimulated goldfish kidney leukocyte supernatants were performed. To demonstrate that stimulated kidney leukocytes were responsible for the production of the NO-inducing factor(s), culture flasks in the absence of stimulated kidney leukocytes were incubated overnight with medium containing serum (10% FCS and 2.5% carp serum). After 16-18 h, mitogens were added as described above and after 6 h the mitogens and serum were removed with three exchanges of HBSS. Samples were removed from the flasks at times indicated above, and were examined by Western blot and the NO bioassay. Samples were also prepared in the absence of mitogen stimulation to test the effect of mixed lymphocyte reactions (MLR) alone on production of NO-inducing factor(s). Finally, the NO-inducing activity of mitogen-stimulated leukocyte supernatants was also examined by generating the supernatants in the presence of FCS (10%) or carp (5%) serum alone. Finally, for some experiments, following mitogen stimulation, leukocytes were incubated in incomplete culture medium supplemented with 25 μg/ml bovine transferrin purchased from Sigma.

2.8 Biochemical characterization of a leukocyte-derived goldfish macrophage deactivation factor (MDF)

2.8.1 Gel permeation fast performance liquid chromatography (GP-FPLC)

Prior to GP-FPLC, crude mitogen-stimulated leukocyte supernatants were concentrated ~30-fold using polyethylene glycol flakes (Sigma) and 3 kD cut-off dialysis membranes (Pierce). The concentrated leukocyte supernatants were then separated according to size using either a Superose 6 or Superose 12 column (Pharmacia). Concentrated samples were centrifuged at 19,000 x g for 10 min. or filtered using a 0.22 μm syringe filter (Millipore) prior to injecting 200 μl samples onto the column. The running buffer used for GP-FPLC was 1x PBS (pH 7.2). All GP-FPLC fractions were collected at 2.5 min. intervals at a flow rate of 0.4 ml/min. into 15 ml centrifuge tubes, and subsequently filter-sterilized (0.22 μm) and stored at –20°C prior to analysis for MDF activities.

2.8.2 Chromatofocusing-FPLC (C-FPLC)

Gel permeation FPLC fractions displaying maximal MDF activity were pooled and concentrated using microcentrifugal concentrators (Filtron; 10 and 3 kD MWCO). Prior to addition of MDF, the polystyrene microcentrifugal sample chamber was blocked for 30 min with 1% calf serum to prevent non-specific absorption of MDF activity. Chromatofocusing of concentrated MDF was performed using a Mono-P column (Pharmacia). The Mono-P column was pre-equilibrated with 0.025

M bis-Tris (pH 7.0, 1M HCL) for establishment of the upper limits of the gradient. A linear descending pH gradient (7.0 - 4.0) was established by running a 1:10 dilution of Polybuffer 74 (Pharmacia) at a flow rate of 0.75 ml/min through the column. MDF samples (500 µl) were allowed to elute through the column for 30 min. prior to initiation of the pH gradient. C-FPLC fractions were collected in 15 ml polystyrene tubes. For biological assays, fractions were collected into 15 ml polystyrene tubes containing an equal volume of 10% calf serum (diluted in 1x PBS) in order to stabilize biological activity.

Proteins bound to the Mono-P column (i.e. proteins having an isoelectric point of less than 4.0) were eluted from the column using a 2M NaCl solution. This salt solution was injected onto the Mono-P column (5 injections of 500 µl) at a flow rate of 0.25 ml/minute. Protein elution was monitored by UV absorption (280 nm), and approximately 2 ml of eluted protein was collected. One ml of the eluent was stabilized by adding an equal volume of 10% calf serum (in 1xPBS), and placed in a dialysis bag (3.5 kD cutoff, SpectroPor). This sample was dialyzed overnight in 1x PBS to remove excess salt. The serum-stabilized SE was subsequently tested for MDF activity using the NO bioassay (Section 2.9.1).

The remaining 1 ml of SE was placed in a serum blocked microcentrifugal concentrator (Filtron; MW cutoff=3 kD) and brought up to a volume of 3 ml with PBS. This sample was concentrated to 50 µl by centrifugation at 7000 x *g*. The sample was subsequently resuspended in an additional 3 ml of 1x PBS, and re-concentrated to 50 µl. This washing step was repeated to ensure complete removal of the salt from the sample. The final 50 µl salt-free sample was stored at -20°C in an equal volume of glycerol prior to examination by SDS-PAGE.

2.9 Functional characterization of a goldfish leukocyte-derived MDF

2.9.1 MDF-mediated inhibition NO production in activated goldfish macrophages

To assess the ability of MDF to inhibit NO production of activated macrophages, 8-10 day old IVDKM were seeded into the wells of half-area 96-well culture plates (Costar) at 5 x 10^4 cells/well and pre-treated for 6 h with GP-FPLC fraction containing maximal MDF activity (1:3 dilution), or C-FPLC salt eluent (1:4 to 1:16). Macrophages were subsequently activated with crude MAF (1:4) and LPS (1 µg/ml), LPS alone (1 and 10 µg/ml), or infected with the obligate intracellular protozoan parasite *Leishmania major* (2 promastigotes to 1 macrophage). Activated macrophages were then incubated for an additional 72 h at 20°C before determination of nitrite production by the Griess reaction (Section 2.25.1). Note: *Salmonella typhirium* lipopolysaccharide (LPS) purchased from Sigma was used throughout this thesis.

2.9.2 Effects of activation sequence, temperature, and MDF dose on NO production in goldfish macrophages

Goldfish macrophages (8-10 days old) were exposed to MDF at varying times pre- and post-activation to determine whether macrophages required pretreatment with MDF in order to deactivate nitric oxide production by activated macrophages. Goldfish macrophages were placed in wells of half-area 96-well culture plates (5 x

10^4 cell/well), and triplicate groups treated with the GP-FPLC fraction with maximal MDF activity (1:3 dilution) 24 or 6 h prior to activation with MAF (1:4) and LPS (1µg/ml). In parallel cultures, macrophages were treated with MAF (1:4) and LPS (1 µg/ml) for 24 h prior to addition of MDF (1:3 dilution). Macrophages were subsequently incubated for an additional 72 h at 20°C before determination of nitrite production by Griess reaction (Section 2.25.1).

Effects of incubation temperature on MDF activity was examined by pre-treating triplicate groups of goldfish macrophages (5 x 10^4 cells/well) with GP-FPLC fraction having maximal MDF activity. Cells were incubated at 20°C or 30°C and subsequently activated with MAF (1:4) and LPS (1 µg/ml). Nitrite production was determined at 24, 48, and 72 h after activation using the Griess reaction (Section 2.25.1).

The effect of MDF dose on inhibition of NO production by goldfish macrophages was determined by plating 5 x 10^4 goldfish macrophages in wells of half-area 96-well culture plates and pre-treating macrophages for 6 h with serial dilutions of MDF (from 1:4 to 1:1024). Macrophages were subsequently activated with MAF (1:4) and LPS (1 µg/ml) or LPS alone (1 and 10 µg/ml) and incubated for 72 h at 20°C before determination of nitrite production.

2.9.3 Effects of MDF on the viability of goldfish macrophages

Goldfish macrophages were seeded in triplicate into 6 ml tubes at a cell density of 2.5 x 10^5 cells in 250 µl. Cells were pre-treated with 125 µl medium, 1X PBS (pH 7.2), or partially purified MDF for 6 h. Macrophages were subsequently activated with MAF (1:4 final dilution) and LPS (1 μg/ml). After a72 h incubation at 20°C, nitrite production was determined in the culture supernatants and cell number and viability was assessed by haemocytometer after staining with trypan blue (Gibco).

2.10 Functional characterization of an exogenous goldfish macrophage deactivation factor (MDF)

2.10.1 Effect of potassium channel blocker treatments on the production of NO and respiratory burst products in goldfish macrophages

Goldfish macrophages (IVDKM) (5×10^4/well) were seeded into 96-well half-area tissue culture plates (Costar) in 50 μl of complete medium. Twenty-five μl of different potassium channel blockers were added to wells in triplicate: 4-AP, or quinine, or TEA at a final concentration of 2.5 mM, 200 μM, and 10 mM, respectively. Macrophages were subsequently stimulated with 25 μl of LPS (10 μg/ml final), MAF (1:4) or MAF (1:4) and LPS (1 μg/ml final) and incubated for 72 h at 20°C prior to determination of nitrite production. A control group was incubated in medium without potassium channel blockers and treated with LPS and/or MAF. For some experiments, a time-course of nitrite production was determined in the absence or presence of potassium channel blockers. Cells were seeded, treated with the blockers, and then stimulated with 10 μg/ml LPS. Nitrite production was determined using the Griess reaction (Section 2.25.1) after 12, 24, 48, and 72 h incubation at 20°C.

Dose-dependent inhibition of nitrite production by potassium channel blockers was determined by seeding goldfish macrophages (5×10^4 /well) into half-area 96-

well culture plates (Costar) and treating triplicate cultures with a range of doses of 4-AP (0mM to 2.5 mM), quinine (0μM to 200 μM) or TEA (0 mM to 10 mM). Cells were simultaneously stimulated with LPS (10 μg/ml) and incubated for 72 h at 20°C prior to determination of nitrite production using the Griess reaction (Section 2.25.1).

The production of ROI in the presence of potassium channel blockers was also determined by seeding goldfish macrophages (5 x 10^4 /well) into 96-well half-area culture plates (Costar) and treating quadruplicate cultures with 4-AP (2.5 mM) followed by priming with MAF (1:4) and LPS (1 μg/ml). Cells were incubated for 6 and 24 h at 20°C prior to determination of ROI production using the NBT reduction assay or the DHR oxidization assay (Section 2.25.2). Cells were also simultaneously examined for relative changes in V_m (Section 2.10.2).

2.10.2 Effect of potassium channel blockers on goldfish macrophage membrane potential (V_m)

To determine the effects of potassium channel blockers on relative changes of macrophage plasma membrane V_m, the fluorescent anionic dye bis-oxonol (Molecular Probes) was used. This negatively charged dye is excluded by the mitochondria and prevents complications of signals derived from mitochondrial origin [517]. Cells were seeded in triplicate (2.5 x 10^5 /tube) into 5 ml polypropylene tubes (Falcon) in 250 μl of medium, and treated with 125 μl of medium (control) or 125 μl of 4-AP (2.5 mM final), quinine (200 μM final), or TEA (10 mM final). Final volume in all tubes was 500 μl with a final concentration of 2% FCS. After 30 min, 2.5 h, and 5.5 h, cells were loaded with 5 μM of bis-oxonol and incubated for a further 25 minutes at 20°C. Cells were then washed 2x with 3 ml medium (control) or with medium supplemented with potassium channel blockers to remove residual bis-oxonol. Cells were re-suspended in a final volume of 250 µl and 50 µl (5 x 10^4 cells) were plated in triplicate into wells of half-area 96-well culture plates (Costar). The relative fluorescence intensity per 5 x 10^4 goldfish macrophages was determined (excitation 544 ± 15 nm; emission 590 ± 14 nm; 60 ms integration time) using a fluorescence microplate spectrophotometer (Molecular Dynamics *fmax*).

2.10.3 Reversibility of altered goldfish macrophage V_m by removal of potassium channel blockers

To determine if the potassium channel blockers used in this study were directly responsible for alterations of goldfish V_m, potassium channel blocker wash-out experiments were performed. Briefly, cells were seeded and incubated with 2.5 mM 4-AP or 200 µM quinine for 3 h. Prior to the addition of 5 µM bis-oxonol, the blockers were removed from the wash-out group by washing the cells 2x with 3 ml medium. After washing, the cells were loaded with bis-oxonol as indicated above, incubated 25 min at 20°C, washed 2x with 3 ml medium to remove bis-oxonol and resuspended in 250 µl of medium. Fluorescence (FL-2) was determined using flow cytometry (FACS Calibur flow cytometer; Becton Dickinson). Flow cytometry was performed by collecting 10,000 cells during analysis with the following settings; forward side scatter-photodiode set to E-1, AmpGain set to 9.33; and side scatter-photomultiplier voltage set to 455 V, AmpGain set to 1.00.

2.10.4 Effects of potassium channel blocker treatment on the viability of goldfish macrophages

Goldfish macrophages were seeded in triplicate into 6 ml tubes at a cell density of 3.0 x 10^5 cells in 250 µl. Cells were then treated with the potassium channel blockers 4-AP, TEA and quinine at final concentrations of 2.5 mM, 10 mM, and 200 µM respectively. Macrophages treated with medium alone were used as the control group. Cells were incubated for 24, 48, and 72 h at 20°C, and at each time point cell number and viability of triplicate cultures was assessed by trypan blue staining and enumerating cells using a haemocytometer.

2.10.5 Analysis of goldfish macophage iNOS mRNA expression following treatment with potassium channel antagonists

Goldfish macrophages were seeded into 5 ml polypropylene tubes (Falcon) (2.5 x 10^6 /tube) in 250 µl of complete medium. Cells were stimulated with 125 µl LPS (10 µg/ml final concentration) and treated with 125 µl medium (control) or 125 µl 4-AP (2.5 mM final concentration) for a final volume of 500 µl. Total RNA was isolated from the cells after 0, 2, 4, 6, 12 and 24 h incubation at 20°C and stored at –80°C prior to performing non-isotopic Northern blot analysis (Section 2.22.7).

2.11 Biochemical characterization of a goldfish kidney leukocyte-derived macrophage NO-inducing factor

2.11.1 Gel-permeation (GP-FPLC) separation of mitogen-stimulated goldfish kidney leukocyte supernatants

Crude mitogen-stimulated goldfish kidney leukocyte supernatants were concentrated and filter-sterilized as described above prior to injecting 200 μl samples onto a Superose 12 size exclusion column (Pharmacia). GP-FPLC was carried out at room temperature using a FPLC system from LKB (Pharmacia, Bromia, Sweden). The running buffer used was PBS (pH 7.2) and all GP-FPLC purification was carried out at flow rates of 0.4 ml/min. Individual fractions were collected at 2.5 min intervals, filter-sterilized and stored at 4°C prior to further analysis.

2.11.2 Chromatofocusing-FPLC (C-FPLC) separation of mitogen-stimulated goldfish kidney leukocyte supernatants

Active NO-inducing fractions obtained from GP-FPLC were subsequently fractionated based on their isoelectric points using C-FPLC. Approximately 4 ml of GP-FPLC purified NO-inducing fractions were concentrated using microcentrifugal concentrators with a 3kD cut-off (Filtron). The polystyrene sample chamber was first blocked for 30 min at room temperature using 10% calf serum to prevent non-specific absorption of proteins to the polystyrene sample chamber. Calf serum was diluted in either 0.25 M Tris- pH 7.5, or 0.75M Tris- pH 9.3 depending on the pH gradient used for fractionation (see below). The chamber was washed extensively with Milli-Q-water prior to addition of GP-FPLC purified fractions.

C-FPLC was carried out at room temperature using a Mono-P column (Pharmacia). Proteins were fractionated by chromatofocusing using two different pH gradients: pH 7.5 - 5.0 or pH 9.3 - 6.0. The Mono-P column was pre-equilibrated in 0.025M bis-Tris (balanced to pH 7.5 using 1M HCl) or 0.075M Tris (balanced to pH 9.3 using glacial acetic acid), for establishment of the upper limits of these gradients, respectively. A descending linear pH gradient of 7.5 - 5.0 was established by running a

1:10 dilution of Polybuffer 74 (Pharmacia) at a flow rate of 0.75 ml/min through the column. A descending linear pH gradient of 9.3 - 6.0 was established by running a 1:10 dilution of Polybuffer 96 (Pharmacia) at a flow rate of 0.75 ml/min through the column. Samples loaded onto the Mono-P column were allowed to elute through the column for 30 min before initiation of the pH gradient.

C-FPLC fractions were collected in tubes containing an equal volume of 10% calf serum (diluted in PBS), in order to stabilize biological activity of purified NO-inducing factor. Sub-samples were also collected in the absence of serum for analysis by SDS-PAGE and Western blot. Nitric oxide-inducing factor activity of the samples was subsequently determined using the Griess reaction (Section 2.25.1).

2.11.3 Ion-exchange chromatography (Mono-Q) separation of mitogen-stimulated goldfish kidney leukocyte supernatants

Ion-exchange chromatography was performed using a Mono-Q column (Pharmacia), which was pre-equilibrated with 20 mM Tris-HCl (pH 9.3) running buffer. Multiple injections of concentrated mitogen-stimulated leukocyte supernatants or GP-FPLC active fractions (10-20 injections of 500 µl/injection) were applied to the column at a flow rate of 0.75 ml/min and proteins that did not bind to the column were collected as a flow-through fraction. Prior to the final injection, 3-4 column volumes of the running buffer were passed through the column to ensure that all unbound proteins were removed. The final injection of concentrated sample was then applied to the column and 5 ml of running buffer was allowed to pass through the column prior to the establishment of a linear salt gradient. The linear ascending salt gradient was established by running 0.5 M NaCl in 20 mM Tris-HCL (pH 9.3) at a flow rate of 0.75 ml/min through the column. Mono-Q fractions were collected in 15 ml polystyrene tubes, filter-sterilized and incubated at 4°C until further analyzed.

2.12 Mass spectrometry analysis of an FPLC-purified goldfish kidney leukocyte-derived NO-inducing factor

The FPLC-purified proteins that induced NO-production in goldfish macrophages were sent to Harvard Microchemistry Facility for analysis by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry on a Finnigan LCQ quadrupole ion trap mass spectrometer. The data were then compared to a database of known proteins to give a preliminary amino acid identification of the purified proteins.

2.13 Generation of a goldfish macrophage anti-NO-inducing factor polyclonal antibody (8C2)

The C-FPLC-purified leukocyte-derived NO-inducing factor was used as a source of antigen for the generation of an anti-NO-inducing factor polyclonal antibody designated as 8C2. The sample used for the primary inoculation of the rabbit was composed of 100 µl volumes of C-FPLC purified fraction (containing the 50 kD NO-inducing factor) plus 150 µl of 1xPBS (sterile) and 300 µl of Freund's Complete Adjuvant (Gibco). After 6 weeks, the rabbit was given a booster composed of identical volumes of antigen, PBS and Freund's Incomplete Adjuvant (Gibco), described above. The production of antibodies was assessed by the reactivity of the rabbit serum with crude mitogen-stimulated leukocyte supernatants using Western blot (Section 2.24).

2.14 Purification of carp serum transferrin

Procedures used for the purification of human serum transferrin were modified for the purification of carp transferrin [518]. Briefly, carp serum was subjected to chromatography on DEAE-Affi-Gel Blue (BioRad) that was equilibrated with 20 mM Tris, 25 mM NaCl, pH 8.0 (running buffer). It was demonstrated that all serum proteins with the exception of transferrin and immunoglobulins bind to the column under these conditions for human serum [518], therefore, carp serum unbound fractions were collected. These fractions were further purified using Con A Sepharose chromatography, protease-digested, and subjected to GP-FPLC and Mono-Q separations using the exact purification protocols described for purification of bovine holo-transferrin described below. Purified proteins were separated by non-reducing SDS-PAGE and the proteins visualized by silver staining (Section 2.24).

2.15 Production of an anti-carp serum transferrin polyclonal antibody (9AG7)

The purified protease-digested carp transferrin was used as a source of antigen for the immunization of rabbits. Primary injection of the rabbit was performed by combining 150 μl of Mono-Q purified digested carp transferrin with 200 μl of 1xPBS (sterile) and 350 μl of Freund's Complete Adjuvant. Booster injections were done exactly as the primary immunizations but substituted with Freund's Incomplete Adjuvant. Specificity of the antibody was determined by reactivity with purified carp serum using Western blot analysis (2.24).

2.16 Isolation of anti-transferrin polyclonal IgG from rabbit serum

The polyclonal anti-carp transferrin IgG prepared in our laboratory [478] cross-reacted with goldfish transferrin and was purified from rabbit serum using a 1ml HiTrap Protein A HP affinity column purchased from Amersham Pharmacia Biotech according to manufacturer's protocols. Briefly, 8 ml of polyclonal rabbit anti-serum was precipitated by the addition of 45% (v/v) ice-cold saturated ammonium sulphate. Serum was allowed to precipitate overnight at 4°C with gentle agitation. Precipitated proteins were removed by centrifugation (5000 x g; 30 min) and re-suspended in 8 ml of 20 mM sodium phosphate, pH 7.0 and dialyzed overnight with 4 L of 20 mM sodium phosphate, pH 7.0 at 4°C in 3 kDa MWCO SnakeSkin dialysis tubing (Pierce). Dialyzed proteins were then concentrated to a volume of 1ml using Macrosep Centrifugal Concentrators (5000xg; 3 kDa MWCO) and the protein concentration of the sample determined using a Micro BCA protein assay reagent kit (Pierce). Twenty mg of precipitated serum protein was then applied to a HiTrap Protein A HP column, which was washed with 10 ml of 20 mM sodium phosphate, pH 7.0. Antibody was then eluted from the column with a decreasing pH gradient (0.1 M citric acid pH 6.0 to pH 3.0) that was neutralized with 1 M Tris-HCL pH 9.0. Each fraction was tested for quantity and purity of isolated IgG using the Pierce protein assay and SDS-PAGE separation. Fractions containing IgG were then pooled, filter-sterilized (0.22 μm filter; Millipore), protein concentration determined, tested for reactivity under native and denaturing conditions and then stored at 4°C until used.

2.17 Immunopurification of goldfish transferrin from crude kidney leukocyte supernatants

Immunopurification of goldfish transferrin was performed using a 1ml HiTrap NHS-activated HP column (Amersham Pharmacia). Affinity A purified anti-carp transferrin IgG (8 mg) was coupled to the column according to the manufacturer's protocols. Mono-Q separated mitogen-stimualted goldfish kidney leukocyte supernatants were used for immunopurification by pooling the fractions that contained maximum NO-inducing activity. These fractions were previously shown to also contain maximum amounts of transferrin cleavage products [478]. All samples applied to the immunoaffinity columns were dialyzed overnight at 4°C against 4 L of column binding buffer (0.05 M Tris-HCL, 0.15 M NaCl. pH 7.6) and adjusted to a final volume of 1 ml by PEG concentration (described above) and designated as pre-affinity samples. Tween 20 (0.05%) was excluded from the binding buffer to prevent reduced viability of macrophages during bioassay.

Prior to sample application, a blank run was performed to ensure that any loosely bound ligand from previous experiments was not present. Briefly, the column was washed with 3 ml of binding buffer followed by 3 ml of elution buffer (0.5 M acetic acid, pH 3.4) and then equilibrated with 10 ml of binding buffer. Samples (Mono Q fractions; 1ml) were then applied to the column and the column was sealed and incubated for 30 min at RT to allow for ligand binding. Unbound proteins were washed from the column with 6 ml of binding buffer and collected into a 15 ml conical tube (Sarstedt). Bound proteins were eluted from the column with 4.5 ml of elution buffer that was neutralized by the addition of 1.5 ml of 1M Tris-HCL, pH 9.0. Unbound and bound samples were immediately dialyzed overnight against 4 L of 1 X

PBS, pH 7.0 then concentrated ~20 fold using Macrosep Centrifugal Concentrators (5000xg; 3 kDa MWCO) and filter-sterilized (0.22 µm) prior to examination by SDS-PAGE/Western blot or used in biological assays. All the applications of samples and buffers was performed using 1ml and 3ml syringes (Becton Dickinson), respectively, at a flow-rate of ~0.5 ml/min.

To test for the activity of immunopurified samples, goldfish macrophages (5 x 10^4 cells/well) were seeded into 96-well half area culture plates in 50 µl of medium supplemented with 5% carp serum (CS). Twenty five µl of pre-affinity, unbound, and bound samples were then added to triplicate macrophage cultures and 25 µl of LPS was also added so that the final stimulatory concentration was 1 µg/ml. Macrophages were incubated for 72 h at 20°C prior to the determination of nitrite production using the Griess reaction (Section 2.25.1).

2.18 Detection of tranferrin-cleaving enzyme activity in goldfish kidney leukocyte supernatants and cellular lysates

Detection of transferrin cleaving activity was performed by incubating 2.5 µg of commercially purchased bovine transferrin (Sigma) with various supernatants believed to contain cleaving enzymes (described below). Transferrin-cleavage was subsequently detected by Western blot analysis using 8C2 (anti-bovine transferrin; 1:400 v/v) as the primary antibody. This strategy allows for the identification of native bovine transferrin exposed to fish cell supernatants or lysates since this antibody does not cross-react with goldfish transferrin [478]. Prior to performing the cleavage assays, 500 µg of transferrin was separated on a Superose12 size exclusion column to remove transferrin breakdown products that we have observed to be present in these purchased preparations. Fractions containing the pure 'native' transferrin were pooled and the protein concentration determined.

Time-course transferrin cleaving activity of the enzymes present in these supernatants was determined by adding 50 µl of crude MAF to 50 µl of medium containing 2.5 µg of transferrin. Samples were then incubated at 20°C for 15 min, 30 min, 1 h, 2 h, 3h, and 4 h (short exposures) and 24, 48 and 72 h (long exposures) prior to removal of 15 µl from each sample and separation by SDS-PAGE and detection by Western blot. A 0 min control was included that represents the addition of 50 µl of crude MAF to 50 µl of medium containing 2.5 µg of transferrin. Fifteen µl of this sample was immediately removed from the control mixture and analyzed.

During the preparation of mitogen-stimualted goldfish kidney leukocyte supernatants, I observed a significant proportion of cellular lysis following mitogen-stimulation (Chapter 6). Therefore, I tested whether products released from damaged/lysed cells contained transferrin-cleaving activity. Kidney leukocytes, cultured macrophages, or kidney granulocytes were washed twice in incomplete medium to remove serum and seeded into separate 6 ml polypropylene tubes (5 x 10^6 cells/tube) in 1ml of incomplete medium. Cells were then lysed with three cycles of freeze-thaw (-80°C for 15 min; 42°C for 5 min) and cellular debris removed by centrifugation (1200 x g, 15 min). Supernatants were collected, filter-sterilized (0.22 µm syringe filter; Millipore) and used immediately in cleaving assays or stored at –20°C. Cleaving activity of the cell lysates was determined by incubating 50 µl of sample with 50 µl of medium containing 2.5 µg transferrin for 24 h followed SDS-PAGE and Coomassie blue staining (Section 2.24).

2.19 Estimation of a potential proteolytic cleavage-site(s) required for the 'activation' of transferrin

To estimate the position of a potential transferrin cleavage site(s) required for producing active fragments, immunopurified goldfish transferrin (Section 2.17) containing macrophage-activating activity were sent to the UVic-Genome BC Proteomics Centre located on the University of Victoria campus (www.proteincentre.com). Q-TOF-Nanospray-tandem mass spectrometry (PE SCIEX API Pulsar) and peptide mapping of trypsin-digested samples were used to confirm the identity of the immunopurified proteins as goldfish transferrin. MALDI-TOF mass spectrometry (PE Biosystems Voyager DE STR MALD-TOF) was also performed on the samples to determine the molecular masses of the individual proteins present in the immunopurified sample. Sizes of the transferrin fragments were then used to estimate the approximate cleavage site by calculating the masses of the fragments by adding the individual masses of each amino acid present in each fragment until the approximate size estimated by mass spectrometry was reached (see Chapter 6).

2.20 Transferrin experiments using commercially purchased transferrin

2.20.1 Protease-digestion and purification of bovine holo-transferrin

Bovine holo-transferrin (iron-saturated) was purchased from Sigma Chemical Co. and processed according to the protocols outlined by [519]. Samples were prepared by dissolving 10 mg of bovine holo-transferrin in 1.5 ml of Concanavalin A buffer (50 mM sodium acetate, 1mM $CaCl_2$, 1mM $MgCl_2$, and 1mM $MnCl_2$ (pH 6.9)). Dissolved transferrin was applied to a Con A-Sepharose column (Sigma) which was pre-washed with 5-10 column volumes of buffer. Unbound transferrin (i.e. non-glycosylated) was collected in a 10 ml flow-through fraction. Bound transferrin (i.e. glycosylated) was eluted from the column by the application of 0.2 M methyl-α-D-mannopyrannoside (Sigma) and collected in a 10 ml fraction. The glycosylated form of the purchased bovine transferrin constituted 70-80% of the total protein. The bound transferrin fraction was subsequently dialyzed overnight against 4 L of 0.1 M Tris-HCl, 25 mM $CaCl_2$ (pH 8.2) then digested with protease type XXVII (Sigma) for 20 h at room temperature (12.5 µg protease/mg of protein). Protease activity was inhibited with the addition of PMSF (Sigma) at 40 µg/mg protease and the digested transferrin was dialyzed overnight against 4L of PBS (pH 7.4), filter-sterilized (0.2 µm filter, millipore) and stored at 4°C. (Note: all of the dialysis steps mentioned above were performed using 3 kD MW cut-off dialysis membranes, Pierce).

2.20.2 FPLC purification of digested bovine holo-transferrin

The purification protocols used for the purification of the goldfish macrophage NO-inducing factor from mitogen-stimulated goldfish kidney leukocyte supernatants were used for the purification of commercially available bovine transferrin. Briefly, precursor (undigested) and protease- digested, Con A-purified, bovine transferrin were separated by non-reducing SDS-PAGE and analyzed by Western blot (Section 2.24) to confirm that the digestion step was successful. Digested transferrin was then separated by GP-FPLC and the individual fractions were once again analyzed by SDS-PAGE followed by Coomassie staining and Western blot. Fractions that contained predominantly digested transferrin (i.e. three protein bands, ~33 kD, ~35 kD, and ~37 kD) were then pooled together and applied to the Mono-Q column for ion-exchange chromatography. Mono-Q fractions were also analyzed by SDS-PAGE and the protein bands visualized by silver staining and Western blot. The effect of Mono-Q separated bovine transferrin fragments on the production of NO in goldfish macrophages was performed by incubating goldfish macrophages ($5x10^4$ cells/well) with individual Mono-Q fractions (1:4 v/v) in conjunction with 1 μg/ml LPS for 72 h at 20°C. Nitrite production was then determined for each fraction using the Griess reaction (Section 2.25.1).

2.20.3 Effect of the addition of bovine holo-transferrin precursor to mitogen-stimulated goldfish leukocyte cultures

Mitogen-stimulated leukocyte supernatants were prepared as outlined above but after an overnight incubation with 5% carp serum only. After mitogen stimulation, the serum and mitogens were removed with three exchanges of HBSS and the cells were incubated for 72 h in either incomplete medium (control) or incomplete medium supplemented with 25 μg/ml of bovine holo-transferrin. Sub-samples (500 μl) were removed from the flasks at 0, 24, 48, and 72 h, filter-sterilized and stored at 4°C. The sub-samples were tested for NO-inducing activity and examined by Western blot (using 8C2 as the primary antibody; 1:400 v/v). Effect transferrin-supplemented mitogen-stimualted goldfish kidney leukocyte supernatants on the production of NO by goldfish macrophages was performed by incubating goldfish macrophages ($5x10^4$ cells/well) with individual Mono-Q fractions (1:4 v/v) in conjunction with 1 μg/ml LPS for 72 h at 20°C. Nitrite production was then determined for each fraction using the Griess reaction (Section 2.25.1).

2.20.4 Effect of transferrin on NO production by goldfish macrophages exposed to different pathogens or pathogen products

Goldfish macrophages (5 x 10^4 cells/well) were seeded into 96-well half area tissue culture plates and stimulated with heat-killed *Trypanosoma danilewskyi* (10:1 parasite to macrophage ratio), heat-killed *Aeromonas salmonicida* (1/200 dilution of heat-killed stock), LPS (1 μg/ml), *Leishmania major* promastigotes (10:1), or *Mycobacterium chelonei/marinum* complex (10:1). *T. danilewskyi* and *L. major* promastigotes were cultivated in our laboratory as previously described [520, 521]. *M. chelonei* was a kind gift from the Provincial Laboratory, University of Alberta, and was grown on 7H10 agar slants or in 7H9 broth at 30°C. Stimulated macrophages were simultaneously incubated in medium alone (control), or medium supplemented with 7.5 μg/well bovine transferrin. Cells were incubated for 72 h at 20°C prior to determination of NO production using the Griess reaction (Section 2.25.1).

2.21 Digestion of transferrin by elastase and induction of NO production in macrophages

Porcine elastase (Roche) was tested for its ability to, a) cleave transferrin and other host proteins (i.e. human fibrinogen and human fibronectin (Sigma); and b) induce NO production in macrophages. To assess the cleaving abilities of porcine elastase, 0.1 μl of normal goldfish serum, 1 μg of recombinant goldfish transferrin expressed in insect cells (Section 2.23.2), 2.5 μg of bovine apo- or holo-transferrin, or

1 μg of human fibrinogen or fibronectin were incubated with 2 μg of elastase or and equal volume of 1xPBS for 2 h at room temperature. Following incubation, samples were separated by SDS-PAGE and stained with Coomassie blue or analyzed by Western blot (Section 2.24) to detect the presence of cleavage products.

Induction of NO production in macrophages by elastase alone or in combination with host proteins was determined by seeding ($5x10^4$ cells/well) macrophages (goldfish or murine P388D.1 cell line) into the wells of 96-well half-area tissue culture plates (Costar). Cells were then treated in triplicate with porcine elastase (10 μg for goldfish macrophages and 5 μg for P388D.1 cells) alone or in combination with 1 μg of insect cell expressed recombinant goldfish transferrin, 10 μg of human fibrinogen, or 10 μg of human fibronectin. Cells were also treated with the host proteins alone without the addition of enzyme. For some treatments, 10 mM of the enzyme inhibitor, PMSF (Sigma), were also added. Nitrite production was determined after a 72 h incubation at 20°C for goldfish macrophages or after 24 h at 37°C for P388D.1 cells using the Griess reaction (2.25.1).

2.22 Goldfish gene expression analysis

2.22.1 Total RNA isolations

Goldfish tissues (i.e. liver, kidney, heart, and spleen), freshly isolated goldfish kidney leukocytes or cultured goldfish macrophages were used for the isolation of mRNA and generation of cDNA templates. Goldfish tissues were aseptically removed and total RNA extracted from 50 μg of the individual tissues using TriZol reagent (Gibco), according to the manufacturer's instructions following various treatments.

Cultured macrophages (5x10^{6} cells/tube) were incubated in 6 ml polypropylene tubes and stimulated with MAF (1:4) and/or LPS (1 µg/ml or 5 µg/ml). After 1, 2, 3, 4, 6, 12, and 24 h incubations at 20°C, total RNA was isolated using TriZol. Freshly isolated goldfish kidney leukocytes (5x10^{6} cells/tube) were also incubated in 6 ml polypropylene tubes and stimulated with 10 µg/ml Concanavalin A (Con A, Roche), 10 ng/ml phorbol myrisitate acetate (PMA, Sigma), and 100 ng/ml calcium ionophore A23187 (Sigma). After 0, 3, 6, and 24 h, total RNA was isolated from the cells. RNA samples were stored in DEPC-treated water at –20°C until used for RT-PCR.

2.22.2 Generation of cDNA templates and PCR

Total RNA (2.5 µg) was used for all first-strand cDNA synthesis using RT-PCR (Stratagene) by incubating with 1.5 µl of oligo (dT) primer (100 ng/µl) at 65°C for 5 min. The reaction was cooled at 22°C for 10 min to allow primers to anneal to the RNA after which the following components were added to the reaction in order; 2.5 µl of 10x first strand buffer, 0.5 µl of RNase Block Ribonuclease Inhibitor (40 U/µl), 1.0 µl of 100 mM dNTPs, and 1.0 µl of MMLV-RT (50 U/µl). The reagents were gently mixed and incubated for 1 hr at 37°C. The reaction was terminated by heating at 90°C for 5 minutes and the cDNA stored at –20°C prior to use in PCR reactions.

PCR reactions were used to amplify iNOS, β-actin, transferrin, and Toll-like receptor PCR products using the primers ordered from Gibco (Table 1.1). All amplification reactions were performed by combining the following reagents (Roche) in order into a master mix such that each reaction contained the following; 0.5 µl of

10x dNTPs (10 mM), 2.5 µl of 10x PCR buffer (with 1.5 mM $MgCl_2$), 0.25 µl of Taq DNA polymerase (5 U/µl), and 20.25 µl of sterile H_2O. The master mix was gently mixed and spun briefly, then 23.5 µl was added to each tube followed by 0.5 µl of Forward and Reverse primers (10 µM) for the different genes. Finally, 0.5 µl of the appropriate cDNA template was added and samples placed immediately in a thermocycler for amplification using the following cycling parameters (unless otherwise stated): (1) 94°C, 3 min; (2) 94°C, 45 sec.; (3) 64°C, 45 sec.; (4) 72°C, 1 min and 30 sec.; 28 cycles of step (2) through (4); (5) 72°C, 10 min. Immediately following PCR amplification, 10 µl of the PCR samples were separated run on a 1.2% agarose/TAE gel and stained with EtBr.

2.22.3 Production of a subtracted goldfish macrophage cDNA library using suppressive subtractive hybridization (SSH)

Goldfish macrophages isolated from 20 individual fish were pooled and 2.5 x 10^7 cells were stimulated with MAF (1:4 final dilution) and LPS (5 µg/ml) or were incubated in medium alone (unstimulated) in separate tubes for 14 h at 20°C. mRNA was isolated (Oligotex kit, Qiagen, Valencia, CA) from total RNA (TriZol, Invitrogen) of control and stimulated macrophages. mRNA from control (unstimulated) and stimulated macrophages were subjected to SSH and selective PCR using the PCR-select cDNA Subtraction Kit (Clontech) according to the manufacturer's protocol, which includes an RsaI digestion step to reduce size bias. Amplified, differentially expressed cDNA fragments were sub-cloned into the pCR2.1-TOPO® vector (Invitrogen) and transformed into chemically competent TOP10 *E. coli* (Invitrogen). Approximately 300 randomly-selected clones were then checked for homologies in the GenBank database using NCBI's BLASTX sequence comparison software at the NCBI website (www.ncbi.nlm.nih.gov/BLAST).

2.22.4 Identification of a goldfish macrophage TLR and sequencing of full-length cDNA using 5'- and 3'-RACE

Screening of the goldfish macrophage SSH cDNA library resulted in the identification of several genes likely to be present in activated cells (data not shown). In particular, a 412 bp fragment was found to have significant homology with the mammalian Toll/interleukin-1 (TIR) domain of the TLR gene. A full-length sequence of this clone was obtained using the SMART RACE cDNA Amplification Kit (BD Biosciences) according to the manufacturer's protocol. Briefly, 5'- and 3'-RACE-Ready cDNA was generated from 500 ng of mRNA extracted from MAF/LPS stimulated goldfish macrophages. 5'-RACE was performed as per manufacturer's protocol using an antisense gene-specific primer based on the EST sequence (Table 1.1). 3'-RACE was performed as described above for 5'-RACE but a sense GSP was used (Table 1.1). Primers were designed to allow for 389 bp of overlap between the 5'- and 3'-RACE products for assembly.

The fragments generated by 5'- and 3'-RACE were sub-cloned into the pCR®2.1-TOPO® vector and sequenced. A single 5'-RACE subclone (2890 bp) was sequenced using the GPS-1 Genome Priming System (New England Biolabs). Two 3'-RACE product of ~1500 bp and ~4500 bp were also sequenced. Sequence fragments were assembled using Chromatool (Biotools, inc.). Each region of the cDNA was sequenced at least three times.

2.22.5 *In silico* analyses

Sequence manipulations were performed using the Genetool (Biotools inc.) and Peptool (Biotools inc.) software packages. Sequence alignments were performed using CLUSTAL W Version 1.81 [522]. Sequences for phylogenetic tree generation and amino acid sequence identity were taken from Genbank (unless otherwise noted) and have the following accession numbers: *Drosophilia* Toll (NM_079794), Urchin Toll (AF335484), goldfish TLR described herein (AY162178), fugu predicted TLR sequence from genome database (www.fugubase.org; SINFRUT00000058729), human TLR9-A (AF259262), mouse TLR9 (AF314224), human TLR8-1 (NM_016610), mouse TLR8 (NM_133212), human TLR7 (AF245702), mouse TLR7 (NM_133211), chicken TLR2 (BAB16843), human TLR2 (NM_003264), mouse TLR2 (NM_011905), mouse TLR1 (AF316985), mouse TLR6 (AF314636), human TLR10 (AF296673), human TLR1 (NM_003263), human TLR6 (NM_006068), human TLR5 (U88881), mouse TLR5 (NM_016928), human TLR3 (NM_003265), mouse TLR3 (AF420279), human TLR4-A (NM_138554), and mouse TLR4 (NM_021297). The TIR domains (as predicted by NCBI conserved domain search) of each accession were used for the phylogenetic tree generation, and gaps were included in the analysis. NCBI BLAST searches were performed for sequence comparison and identification of conserved homologous regions. Motif Scan (http://hits.isb-sib.ch) was used for identification of conserved protein motifs. Phylogenetic tree construction was performed using TREE-PUZZLE [523], which constructs trees of sequence based on maximum likelihood using a quartet puzzling algorithm. Signal peptides, transmembrane regions, and molecular weights were predicted using SOSUI [524] (http://sosui.proteome.bio.tuat.ac.jp).

2.22.6 Isotopic Northern blot analysis

2.22.6.1 Examination of goldfish macrophage TLR mRNA expression

Northern blot analysis was performed according to a modification of the procedure outlined by Sambrook *et al.,* [525]. Briefly, total RNA (8 µg/lane) was run on a 1% formaldehyde agarose gel, prior to transfer to Nytran membranes (Schleicher and Schuell). Hybridization and prehybridization were carried out at 42°C in buffer consisting of 50% Formamide, 5X SSC, 5X Denhardt's reagent, 1% sodium dodecyl sulfate, and 100 µg/mL sheared and heat denatured calf thymus DNA. A ^{32}P-radiolabeled goldfish TLR probe was prepared using a 2586 bp RT-PCR product isolated from activated goldfish macrophage cDNA template (Section 2.22.2) using the TLR primers listed in Table 1.1. The goldfish macrophage TLR PCR product was then labeled using a standard random primed labeling protocol [525]. Relative expression levels of the ~3.5kb band were determined by densitometry using the Kodak ID 3.0 software (Eastman Kodak) and normalized to unstimulated control expression levels.

2.22.7 Non-isotopic Northern blot anlysis of iNOS mRNA expression in goldfish macrophages exposed to potassium channel blockers

2.22.7.1 Generation of PCR products for non-isotopic probes

iNOS and β-actin PCR products were generated as described in Section 2.22.2. Eight µl of the PCR sample was run on a 1.6% agarose/TAE gel for confirmation of the size of PCR products. The remainder of the PCR products were run through GFX™ PCR DNA Purification Columns (Pharmacia) to remove unincorporated nucleotides and excess salts. The purified PCR products were re-suspended to a final concentration of 50 ng/µl and stored at –20°C prior to non-isotopic labeling.

2.22.7.2 Non-isotopic labeling

The purified PCR products were labeled using the BrightStar™ Psoralen-Biotin Non-isotopic Labeling Kit (Ambion) according to the manufacturers protocols. Briefly, 10 µl (500 ng) of PCR products were heat denatured for 10 min at 100°C followed by immediate quick-chill by placing the samples in an ice-water bath. Freshly denatured DNA samples were labeled by the addition of 1 µl of psoralen-biotin reagent in the well of a microtitre plate (Costar). The plate was placed on ice and irradiated with a UV lamp set to 365 nm for 45 min. The sample was then diluted up to 100 µl by the addition of 89 µl of 1x TE buffer and transferred to a clean microcentrifuge tube. Non-cross linked Psoralen-Biotin reagent was removed by two rounds of butanol extractions and the non-isotopic labeled probes were aliquoted into

20 µl samples and stored at –80°C. The final concentration of the probes was approximately 5 ng/µl.

2.22.7.3 Non-isotopic detection

Northern blot analysis was performed using the NorthernMax™ kit (Ambion) according to the manufacturers protocols. Briefly, RNA samples (10 µg/lane) were diluted with 3 volumes of formaldehyde load dye (Ambion) and incubated at 65°C for 15 min to denature RNA secondary structure. Samples were then loaded onto a 0.6 mm thick, 1% formaldehyde agarose gel and electrophoresed for 1.5 h at 125 volts. Following electrophoresis, RNA was transferred to Ambion's BrightStar-Plus™ positively charged nylon membranes using a downward transfer assembly. The RNA was cross linked in a UV Stratalinker 2400 (Stratagene) at 120,000 µJ, and the membrane pre-hybridized for 1 h at 42°C using ULTRAhyb™ hybridization solution provided by Ambion. Non-isotopic probes (e.g. iNOS and b-actin) were heat denatured by diluting in 50 µl 10 mM EDTA and incubating at 90°C for 10 min and added directly to the appropriate pre-hybridized membrane. Probe and membrane were allowed to hybridize overnight (16 h) at 42°C with gentle agitation. Hybridization solution was removed and the membranes were washed with two 5 min washes with 2X SSC/0.1 % SDS (low stringency) at room temperature followed by two 15 min washes with 0.1X SSC/0.1% SDS (high stringency) at 42°C. Membranes were briefly blotted to remove excess wash solution and RNA expression detected using the BrightStar™ BioDetect™ non-isotopic detection kit (Ambion) following the manufacturer's protocols. The non-isotopic labeled probes were visualized using a chemiluminescent detection system followed by exposure of the membrane to film (Kodak) at room temperature.

2.22.8 Southern blot analysis of a goldfish macrophages TLR

Goldfish genomic DNA was prepared from testes and muscle by the method of [525]. DNA aliquots were digested overnight with each of Pst I, Hind III, Bam HI, and Sph I. Sph I is the only one of the four restriction enzymes predicted to cut within the coding region of the goldfish TLR. Fifteen µg of each digest was loaded per lane and following electrophoresis digests were transferred to Biodyne B nylon membrane (PALL) for hybridization. Pre-hybridization and hybridization were carried out at 42°C in buffer consisting of 50% Formamide, 5X SSC, 5X Denhardt's reagent, 1% sodium dodecyl sulfate, and 100 µg/mL sheared and heat denatured calf thymus DNA. A ^{32}P-radiolabeled goldfish TLR probe was prepared using the 2586 bp RT-PCR product isolated from reactions described above, using a standard random primed labeling protocol [525]. Washes were carried out at low stringency and culminated in a final wash of 0.5X SSC/1% SDS at 37 °C. Membrane was exposed to Kodak X-Omat-AR X-ray film for 18 h at -70 °C prior to development.

2.22.9 DNA Sequencing

All DNA sequencing was performed using the DYEnamic ET-Terminator Cycle Sequencing Kit (Amersham-Pharmacia Biotech, Uppsala, Sweden) and Applied Biosystems 377 DNA sequencers according to standard manufacturer supplied protocols.

2.23 Expression of recombinant goldfish transferrin

2.23.1 Prokaryotic expression

2.23.1.1 Generation of PCR product for cloning into prokaryotic expression vector

Full-length goldfish transferrin and goldfish transferrin lobes (i.e. N-lobe and C-lobe) were expressed in *E. coli* using the pET Directional TOPO® Expression Kit (Invitrogen). Briefly, PCR construct primers were designed using goldfish transferrin cDNA as a template and were designed to amplify the predicted C-lobe of goldfish transferrin (Table 1.1). The nucleotides "CACC" were appendend to the 5' end of the forward expression primer to ensure directional cloning into the pET expression vector. The estimated sizes of full length, C-lobe, and N-lobe goldfish transferrin PCR products were 2100 bp, 1075 bp, and 996 bp, respectively. PCR products were amplified from a goldfish liver cDNA template as described in Section 2.22.2. Cycling parameters for the amplification of goldfish transferrin expression constructs were as follows; 1) 94°C for 3 min; 2) 30 cycles of 94°C for 45 sec, 62°C for 45 sec, and 72°C for 2 min 30 sec; 3) 72°C for 10 min. Ten µl of the PCR product was visualized on a 1.2% agarose/EtBr gel.

2.23.1.2 Cloning and verification of the goldfish prokaryotic expression constructs

One µl of the various PCR products (i.e. Full-length goldfish transferrin, N-lobe, and C-lobe) were cloned into the pET/100 Directional TOPO® Expression Kit (Invitrogen) and transformed into chemically competent TOP10 *E. coli* (Invitrogen) according to manufacturer's protocol. Cells were plated onto LB-Ampicillin (50 μg/mL) plates and incubated for 18 h at 37°C. Positive clones were identified by randomly picking 10 colonies and performing colony PCR using the vector specific primers T7 and T7 Reverse (Table 1.1) supplied by Invitrogen. Positive clones were grown for 18 h in 6 ml LB medium (50 μg/ml ampicillin) and plasmids prepared using a QIAprep® Spin Miniprep kit (Qiagen). Five hundred nanograms of 5 randomly chosen plasmids were then digested with Nhe I and Sac I (New England Biolabs) for 1 h at 37°C and the inserts detected by separating the DNA on a 1.2% agarose/EtBr gel. Each restriction digest consisted of 5 μl plasmid (0.5 μg), 1 μl of 10x Buffer 1, 1 μl BSA, 0.3 μl of Not I and Bam HI, and 2.4 μl H_2O. To verify that the goldfish transferrin expression constructs were cloned into the expression vector in the correct orientation and that the construct was 'in frame', inserts were sequencing using the DYEnamic ET-Terminator Cycle Sequencing Kit (Amersham-Pharmacia Biotech, Uppsala, Sweden) and Applied Biosystems 377 DNA sequencers according to standard manufacturer supplied protocols. The vector specific primers (T7 and T7 Reverse) were used for all sequencing reactions.

2.23.1.3 Transformation and expression of recombinant goldfish transferrin in *E. coli*

Plasmid DNA containing expression constructs were transformed into BL21 Star™ (DE3) One Shot® *E. coli* (Invitogen) for recombinant protein expression. Briefly, 10 ng of plasmid DNA (5 μl total volume) was transformed into the bacteria by heat-shocking at 42°C for 30 seconds. Transformed bacteria were then grown overnight at 37°C in LB medium supplemented with either 100 μg/ml ampicillin or 50 μg/ml carbenicillin prior to pilot expression.

2.23.1.4 Pilot Expression

Induction of recombinant protein expression was performed by the addition of IPTG according to manufacturer's protocols. Briefly, 10 ml of LB medium containing 100 μg/ml ampicillin or 50 μg/ml carbenicillin were inoculated with 500 μl of an overnight culture and allowed to grow for 2 h at 37°C with shaking until they reached mid-log phase of growth (i.e. O.D.$_{600}$ ~0.5-0.8). IPTG was then added to a final concentration of 0.1 mM to 5.0 mM and a 500 μl aliquot was removed from the culture, centrifuged at 14 000 rpm in a microcentrifuge for 30 seconds, and supernatants removed and cell pellets were then frozen at –20°C (these are the 0 h time-point samples). Remaining cultures were incubated at 37°C with shaking and 500 μl aliquots removed after 1, 2, 4, and 6 h post induction. For each time-point, samples were processed as described above. Individual samples were then analyzed by SDS-PAGE and Western blot for the presence of recombinant protein expression (Section 2.23.4).

2.23.1.5 Scale-up expression of recombinant proteins produced in *E. coli*

From pilot expression experiments, it was determined that the optimal conditions for production of recombinant protein in *E. coli* was a 2 h incubation in LB medium containing 50 μg/ml carbenicillin and 0.1 mM IPTG. For large-scale expression and purification of the target protein, 50 ml of LB medium containing 50 μg/ml carbenicillin was grown overnight at 37°C with shaking to an O.D.$_{600}$ of ~1.0-2.0. Ten ml of this culture was then inoculated into 250 ml of LB (50 μg/ml carbenicillin) and a total of four flasks were prepared (1 L total). Cultures were incubated until mid-log phase of growth was achieved followed by the induction of target protein expression with 0.1 mM IPTG. Cultures were then grown for 2 h prior to the purification of the His-tagged recombinant.

2.23.1.6 Harvest and lysis of *E. coli*

E. coli cell cultures (1 L) expressing the protein of interest, were centrifuged at 5000 x g (5500 rpm in a Sorvall GSA rotor) for 15 min. at 4°C in pre-weighed centrifuge bottles. Supernatants were decanted and the wet weight of the *E. coli* cell pellet was determined. Three ml of ice-cold cell-lysis buffer I (50 mM Tris-HCL, 1 mM EDTA, 100 mM NaCl; pH 8.0) was added per gram of *E. coli* and the suspension mixed thoroughly by vortexing. For each gram of *E. coli*, 4 μl of 100 mM PMSF and 80 μl of 10 mg/ml of lysozyme were added and the suspension stirred for 20 minutes. Next, 4 mg of deoxycholic acid was added per gram of *E. coli* and the suspension incubated at 37°C and stirred occasionally with a glass rod until the lysate became viscous followed by the addition of 20 μl of 1mg/ml DNase I per gram of *E. coli*. Finally, lysates were incubated at room temperature with constant agitation until solution was no longer viscous (~30 min) and recombinant proteins were then isolated from inclusion-bodies.

2.23.1.7 Recovery of inclusion bodies using urea

Recovery of inclusion-bodies was performed using a procedure adapted from Schoner *et al.*, [526]. Cellular lysates were centrifuged at 14 000 rpm in a microcentrifuge for 15 min. at 4°C. Supernatants were removed, the pellet resuspended in 1 ml of H_2O per gram of *E. coli* and centrifuged at 14 000 rpm for a further 15 min. Supernatants were discarded and pellet resuspended in 100 µl of 0.1 M Tris-Cl (pH 8.5), containing 1.0 M urea per gram of *E. coli* and centrifuged at 14 000 rpm for 15 min. to isolate the inclusion bodies. Sub-samples of supernatant were removed for analysis by SDS-PAGE and the remaining inclusion-body pellet resuspendend in 100 µl of H_2O per gram of *E. coli* and pelleted by centrifugation at 14 000 rpm for 15 min.

2.23.1.8 Solubilization of inclusion-bodies

Inclusion-bodies were solubilized by adding 100 µl of inclusion-body solubilization buffer I (50 mM Tris-Cl, 1 mM EDTA, 100 mM NaCl, 8M urea, and 0.1 M PMSF; pH 8.0) per gram of *E. coli*. Note: urea and PMSF were added just prior to use. Mixture was allowed to incubate at room temperature for 1 h followed by the addition of 9 volumes of inclusion-body solubilization buffer II (50 mM KH_2PO_4, 1 mM EDTA, 50 mM NaCl; pH 10.7) and solution was incubated for a further 30 min. at room temperature. After 30 min., pH of the solution was adjusted to pH 8.0 with 12 N HCl and incubated for a further 30 min. at room temperature. Finally, solution was centrifuged at 14 000 rpm for 15 min. in a microcentrifuge and supernatants containing recombinant proteins analysed by SDS-PAGE and Western blot and stored at 4°C prior to renaturation.

2.23.1.9 Renaturation of recombinant proteins purified from inclusion bodies

Procedure for the renaturation of recombinant proteins was adapted from Hoefkens *et al.,* [527]. Renaturation was performed by diluting the proteins in renaturation buffer (0.1 mM Na-EDTA, 0.1 mM Tris-Cl, 1.0 mM GSH (reduced glutathione); pH 8.2) to a concentration of 20 µg/ml at 4°C for min. Subsequently, GSSG (oxidized glutathione) was added to a final concentration of 0.5 mM and the solution was incubated for a further 22 h at 4°C. After incubation, the solution was dialyzed against 8 L of 1xPBS pH 7.4 using 3 kD MWCO dialysis membranes (Pierce). Concentration of the recombinant proteins was then determined using the Micro BCA protein assay reagent kit (Pierce). The solution was then filter-sterilized (0.2 µm) and stored at 4°C prior to purification of the recombinant proteins (Section 2.23.3).

2.23.1.10 Removal of LPS and induction NO production in macrophages by recombinant goldfish transferrin lobes

Removal of contaminating LPS was performed by application of samples to a Detoxi-Gel™ endotoxin removal column purchased from Pierce. Briefly, 1 ml columns were regenerated by washing with 5 ml of 1 % sodium deoxycholate (Sigma), followed by 5 column volumes of pyrogen-free water to remove excess detergent. Recombinant samples were then applied to the column (1 ml samples) and allowed to flow-through the column by gravity. Column was allowed to incubate for

1 h at 4°C, and the sample was then eluted by adding sterile 1xPBS. Collected samples were then dialyzed overnight vs. 4L of 1xPBS in 3 kD MWCO dialysis membranes, filter-sterilized (0.2 μm), and stored at 4°C. Detection of contaminating levels of endotoxin was also determined using the Limulus Amebocyte Lysate (LAL) Endosafe® kit according to manufacturer's instructions (Charles River).

Induction of NO production in macrophages (goldfish, P388D.1 cell line, or BMDM) by recombinant goldfish transferrin was determined by seeding cells (5×10^4 cells/well) in 96 well half-area tissue culture plates (Costar). Macrophages were then treated in triplicate with recombinant transferrin alone (37.5 to 300 ng/well) or in conjunction with 1 μg/ml LPS. For some experiments, macrophages were also treated with recombinant protein in conjunction with 10 μg/ml polymixin B sulphate (Sigma). Cells were incubated for 72 h at 20°C (goldfish macrophages) or for 24 h at 37°C (mouse macrophages) prior to determination of NO production using the Griess reaction (Section 2.25.1).

2.23.2 Eukaryotic expression

2.23.2.1 Generation of PCR product for cloning into insect expression vector

Full-length goldfish transferrin was expressed in insect cells using the pIB/V5-HIS TOPO® TA eukaryotic expression kit (Invitrogen). Eukaryotic expression primers (Table 1.1) were used to generate PCR products using cDNA generated from goldfish liver tissue as a template (Section 2.22.2) but with a *Taq* mixture consisting of a 15:1 ratio of excess *Pfu Taq* over non-proofreading polymerase. Cycling parameters for the PCR reaction were as follows; 1) 94°C for 3 min; 2) 30 cycles of 94°C for 45 sec, 55°C for 45 sec, and 72°C for 2 min 30 sec; 3) 72°C for 10 min. Ten µl of the PCR product was visualized on a 1.2% agarose/EtBr gel.

2.23.2.2 Cloning and verification of the goldfish transferrin eukaryotic expression construct

One µl of goldfish transferrin PCR product was cloned into the pIB/V5-HIS® vector (Invitrogen) and transformed into chemically competent TOP10 *E. coli* (Invitrogen) according to manufacturer's protocol. Cells were plated onto LB-Ampicillin (50 µg/mL) plates and incubated for 18 h at 37°C. Positive clones were identified by randomly picking 10 colonies and performing colony PCR using the vector specific primers OPIE2 Forward and OPIE2 Reverse supplied by Invitrogen. Clones containing inserts were grown for 18 h in 6 ml LB medium (50 µg/ml ampicillin) and plasmids prepared using a QIAprep® Spin Miniprep kit (Qiagen). Two µg of 5 randomly chosen plasmids were then digested with Not I and Bam HI (New England Biolabs) for 3.5 h at 37°C and the inserts detected by separating the DNA on a 1.2% agarose/EtBr gel. Each restriction digest consisted of 3 µl plasmid (2 µg), 1 µl of 10x Buffer 3, 1 µl BSA, 0.2 µl of Not I and Bam HI, and 3.6 µl H_2O. To verify that the goldfish eukaryotic expression constructs were cloned into the in the correct direction and that the gene was 'in frame', inserts were sequencing using the DYEnamic ET-Terminator Cycle Sequencing Kit (Amersham-Pharmacia Biotech, Uppsala, Sweden) and Applied Biosystems 377 DNA sequencers according to standard manufacturer supplied protocols. The vector specific primers (OPIE2 Forward and Reverse) were used for all sequencing reactions.

2.23.2.3 Transient expression in insect cells and selection of stable cell lines

The insect cell line Sf9 (Invitrogen), were used for all experiments and were grown in ESF-921 medium (Expression Systems LLC) at 27°C. Insect cells were grown in 25 cm^2 tissue culture flasks and were grown to log phase prior to transfection. In a 6-well tissue culture plate (Costar), $3x10^6$ Sf9 cells/well were incubated in 1 ml Grace's insect medium (Sigma) for 30 min at 27°C to allow cells to attach to plate. During this incubation period, 4 μg of plasmid DNA containing the rLRR expression construct was added to 1 ml Grace's in a sterile 1.5 ml eppendorf tube. Contents were gently mixed and 10 μl of Cellfectin reagent (Invitrogen) added. The tube was then allowed to stand for 30 min for complexes to form. After 30 min, the Grace's medium was gently removed from the insect cells by tilting the plate and sucking the media off with a sterile 1 ml pipette. Then the Grace's/DNA/Cellfectin mixture was gently added to the adhered insect cells and the plate incubated for 4 h at 27°C. Plate was gently rocked every hour to mix the contents and after 4 h, an additional 1 ml of Grace's was added to the cells and the plate incubated for a further 48 h at 27°C. After 48 h, supernatant was removed and stored at 4°C until examined for the presence of recombinant protein as described below.

Five ml of ESF-921 medium was added to the transfected cells and the cells were removed from the bottom of the wells by gently mixing with a pipette tip. Cells and medium were then transferred to a 25 cm^2 tissue culture flask and allowed to attach for 2 h at 27°C. Production of a stable polyclonal cell line was performed by selecting for transfected cells using Blasticidin S HCL (75 μg/ml). Cells were incubated for 4 days in the presence of the selection agent and after 4 days medium was exchanged with fresh ESF-92 with 75 µg/ml Blasticidin. This was repeated every 4 days until a confluent layer of cells was observed. A sample of medium was removed from the confluent cells and assayed for the presence of recombinant protein (see below). Adhered cells were removed by agitation and the cells transferred to either 25 cm^2 tissue culture flasks (500 µl cells into 4.5 ml fresh ESF-921 medium) or 75 cm^2 tissue culture flasks (2 ml cells into 18 ml of fresh ESF-921 medium). The polyclonal cell lines were always maintained in the presence of 10 µg/ml Blasticidin and incubated for 5 days at 27°C prior to passaging. After each passage, supernatants were collected and used for the identification and purification of recombinant proteins (Section 2.23.3).

2.23.3 Purification of recombinant proteins

Renatured recombinant proteins purified from inclusion-bodies or recombinant proteins present in the supernatants of transformed insect cells renatured were collected and dialyzed for 2 days at 4°C against 8 L of NiNTA wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole; pH 8.0) in 3 kD MWCO dialysis membranes (Pierce). Following dialysis, samples were concentrated 10-fold using polyethylene glycol flakes (Sigma). Concentrated samples were then dialyzed overnight against 8 L of NiNTA wash buffer at 4°C and filtered using a 0.8 µm filter.

Using the N- or C-terminal histidine tags inserted onto the recombinant protein by the pET/100 Directional TOPO® and pIB/V5-HIS TOPO® vector, respectively, recombinant proteins were purified under native conditions using

NiNTA agarose purchased from Qiagen. Briefly, 5 ml of settled agarose (binding capacity of 5-10 mg protein per 1 ml of settled matrix) was poured into a 10 cc syringe fitted with a stop-cock. Agarose was washed with 5 volumes of NiNTA wash buffer prior to addition of concentrated/dialyzed samples. Proteins were allowed to flow through the column using gravity until the entire sample had passed-through the 5 ml of agarose. The column was then allowed to stand for 1 h at 4°C before being washed with 5 column volumes of NiNTA wash buffer. Recombinant proteins were then eluted with wash buffer supplemented with 125 mM imidazole and the eluted proteins collected as 1 ml fractions. Samples were then analyzed by SDS-PAGE and Western blot (Section 2.23.4) and the fractions containing purified recombinants were dialyzed overnight against 4 L 1xPBS in 3 kD MWCO dialysis membranes (Pierce), filter sterilized (0.2 µm filter; Millipore) and stored at 4°C prior to use in biological assays.

2.23.4 Detection of recombinant proteins by Western blot

The pET100/D-TOPO® and the pIB/V5-HIS TOPO® vectors incorporated a N-terminal 6xHis-Tag or C-terminal V5 epitope into the recombinant proteins, respectively, which allowed for easy detection using Western blot analysis. Detection of recombinant proteins was performed by separating samples by reducing SDS-PAGE using a 12.5% polyacrylamide gel (Section 2.24). Following SDS-PAGE, proteins were transferred to 0.2 µm nitrocellulose membranes (BioRad) and incubated for 18 h at 4°C with either a 1:5000 (v/v) dilution of the anti-HisG-AP Antibody or a 1:5000 (v/v) dilution of the anti-V5 mAb, purchased from Invitrogen. Membranes were then washed and the protein bands visualized as described in Section 2.24.1 and 2.24.2.

2.24 SDS-PAGE electrophoresis and Western blot analysis

Samples (15 µl) were dissolved in an equal volume of 2X sample buffer (62.5 mM Tris-HCL pH 6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol Blue; for reducing SDS-PAGE, 1/20 dilution of β-ME added to sample buffer), heated at 95°C for 5 min. and electrophoresed through 5% stacked, 12.5% continuous separating polyacrylamide gels. Electrophoresis was carried out in 1x SDS running buffer (25 mM Tris, 192 mM Glycine, 0.1% (w/v) SDS, pH 8.3; Sigma) at 100 V for 12 min followed by 185 V for 40 min. Protein bands were visualized by either staining the gels with 0.04% Coomassie Brilliant Blue R-250 (BioRad) dissolved in 40% methanol/10% acetic acid for 30 min or with a silver staining kit (BioRad).

For Western blot analysis, protein samples were separated by SDS-PAGE as indicated above, and equilibrated in transfer buffer (25 mM Tris-HCL, 192 mM Glycine, 20% (v/v) Methanol, pH 8.3) for 5 min. Protein samples were electroblotted onto 0.2 µm nitrocellulose sheets (BioRad) at 100 V for 1h. After protein transfer, the nitrocellulose was reversible stained with Ponceau S (Sigma) for visualization of the SDS-PAGE molecular weight standards (BioRad). The standards were removed by cutting with a scalpel and the remaining nitrocellulose was washed with milli-Q water to remove excess stain. Nitrocellulose sheets were then placed in blocking buffer (100 mM Tris-HCL, 0.9% (w/v) NaCl, 0.5% (w/v) BSA, and 0.1% Tween 20, pH 7.5) and incubated for 30 min at room temperature. Polyclonal antibodies [i.e. anti-NO-inducing factor (8C2) or anti-carp serum transferrin (9AG7)] were added to the blocking buffer at a 1/400 dilution and the nitrocellulose was incubated on a rocking platform overnight at 4°C. Note: all Western blots performed with 8C2 or 9AG7 were run under non-reducing (i.e. no β-ME) conditions.

2.24.1 Colorimetric Dectection (NBT/BCIP)

Protein bands were visualized using an alkaline phosphatase conjugated goat anti-rabbit IgG (H+L) secondary antibody (BioRad). Briefly, the primary antibodies were removed by extensive washing with Tween 20/tris-buffered saline (TTBS; 0.1% Tween 20 in 100 mM Tris-HCL, 0.9% NaCl, pH 7.5; TBS). The secondary AP-conjugated antibody was added to the nitrocellulose sheet in blocking buffer at a 1:2000 (v/v) dilution. The nitrocellulose was incubated for 1 h at room temperature on a rocking platform. Secondary antibody was removed by extensive washing with TTBS followed by three exchanges with TBS. The bands were visualized using the chromogenic BCIP/NBT development kit according to the manufacturers protocol (BioRad).

2.24.2 ECL Detection

Protein bands were visualized by a light emitting non-radioactive method for the detection of proteins using the ECL Western blotting kit from Amersham Biosciences. Briefly, proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes as described above. Primary antibodies were removed by extensive washing with TTBS, and the secondary Horseradish Peroxidase (HRP) labeled secondary antibodies (anti-mouse or anti-rabbit) were added to the membrane in blocking buffer at a 1:5000 (v/v) dilution. The membrane was incubated for 1 h at room temperature, secondary antibodies were washed away as described above, and protein bands detected by exposure to X-ray film according to the manufacture's instructions.

2.25 Biological assays

2.25.1 Nitric oxide bioassay

Nitric oxide production by activated macrophages was determined indirectly using the Griess reaction [528]. Seventy-five µL of the supernatants from individual macrophage cultures was transferred to a microtitre plate and 100 µL of 1% sulfanilamide (Sigma) (dissolved in 2.5% H_3PO_4) followed by 100 µl of 0.1% N-naphthyl-ethylenediamine (Sigma) (dissolved in 2.5% phosphoric acid) was added to each well. The plate was allowed to sit for 2 min before the optical densities (O.D 540 nm) were determined using an automated microtitreplate spectrophotometer (Biotek). The approximate concentration of nitrite in samples was determined from a standard curve generated using known concentrations of sodium nitrite (Fig. 1.1)

2.25.2 Respiratory burst biossays

2.25.2.1 NBT Reduction

Nitro blue tetrazolium (NBT; Sigma) was dissolved in dimethyl sulfoxide (DMSO; 20%w/v; BDH) to which pre-warmed (50°C) Dulbecco's PBS (Gibco) was added to completely dissolve NBT (final NBT concentration= 2 mg ml/ml). This NBT solution was heated for an additional 10 min at 50°C and subsequently filter sterilized. PMA was added to the NBT solution such that the final triggering concentration within macrophage cultures was 50 ng/ml. Fifty µl of the NBT/PMA solution was added to macrophage cultures and plates centrifuged (200 x *g*, 1 min) to pellet cells. Cultures were subsequently incubated for 25 min at room temperature. Supernatants were subsequently removed and macrophage layers fixed by adding 200 µl of 70% methanol for 1 minute. Unreduced NBT was removed by washing cells several times with 70% methanol. Reduced NBT was dissolved by adding 60 µl of 2M KOH to individual wells and pipetting vigorously. Seventy µl of dimethyl sulfoxide (DMSO) was then added to wells and optical densities read at 630 nm on a microtiterplate spectrophotometer.

2.25.2.2 Dihydrorhodamine (DHR) oxidation assays

The respiratory burst activity of macrophages was measured using a modified procedure used for the analysis of mammalian neutrophil respiratory burst activity [529-531]. DHR (Molecular Probes) was dissolved in DMSO at a concentration of 29 mM (stock solution), and stored in 30 µl aliquots at –80°C. Samples of IVDKM or T-PKM cultures (day 8-12) were ($2.5x10^5$ cells/500 µl) seeded into 6 ml polystyrene tubes (Falcon) and allowed to equilibrate for 1 h at 22°C prior to determination of the respiratory burst response.

Following the 1 h incubation, tubes were centrifuged at 200 x *g* for 10 min. Supernatants were removed and the cells were re-suspended in 2 ml sterile PBS. The wash step was repeated once more to remove all traces of serum. After the final wash, cells were loaded with 100 µl DHR (10 µM final concentration in PBS) and incubated for 5 min at 22°C. The respiratory burst response was triggered by adding 400 µl PMA (100 ng final concentration in PBS) and the cells were incubated for an additional 30 min to allow for the oxidation of DHR to the fluorescent compound Rho 123. Control tubes were loaded with DHR but were treated for 30 min with sterile PBS only. A positive control was included by incubating DHR-loaded cells with 1 mM hydrogen peroxide (Merck) and samples incubated at 4°C (reaction inhibition step) were used as the negative control. After the 30 min priming step, samples were immediately analyzed using a flow cytometer. Instrumental settings for forward and side scatter were the same as described above, and the assessment of green fluorescence (Rho 123) was determined (FL1) using histograms and a logarithmic scale. Cells treated with DHR only (no triggering) were used to calibrate the FL1. Each analysis was performed on 10^4 events and analyzed using the LYSIS II software. The degree of respiratory burst activity was assessed from the mean fluorescence intensity values obtained from the histogram plots. For detection of functional populations, regional analysis was performed on histograms and cytograms using the LYSIS II software.

2.26 Statistical analysis

One and two-way analysis of variance using SuperAnova software (Abacus) for the Power Macintosh was used for determining significance between control and experimental groups. Probability level of $P<0.05$ was considered significant.

Table 1.1 List of primers used throughout thesis[a]

Primer		5' to 3'
iNOS	F	GGAGGTACGTCTGCGAGGAGGCT
	R	CCAGCGCTGCAAACCTATCATCCA
β-actin	F	CGAGCTGCGTGTTGCCCCTGAG
	R	CGGCCGTGGTGGTGAAGCTGTAG
Transferrin 1	F	CCACCGAGCGAGAGGCAGGACTA
	R	CCCTTACGCACCACGGCCACAAC
Transferrin 2	F	GCTCATCTCGTTTCTGGCGTGCC
	R	GATCAGCACCAGGAGCGCAGCC
TLR	F	TTTCAGGATTCCGTCGATACC
	R	GTTCGTTTCTTCACCAGCTTCC
Transferrin (Full-length)[b]	F	CACCAACATCCTGCTCATCTCGT
	R	GGGGGAAACATGCAAGATAAAGCTC
Transferrin (N-lobe)[b]	F	CACCAACATCCTGCTCATCTCGT
	R	CTAAGGTGGAGCTGGTGGGTTCCC
Transferrin (C-lobe)[b]	F	CACCACCATTAGCCATGCAGAG
	R	GGGGGAAACATGCAAGATAAAGCTC
Transferrin (Full-length)[c]	F	ATGATCCTGCTCATCTCGTTTCTG
	R	TGTGCATGCCTTGACCAGATC
TLR 5' RACE	R	GTTCGTTTCTTCACCAGCTTCC
TLR 3' RACE	F	CGACAATAAGAAGAAGCAGAGC
pET Directional TOPO® (T7)	F	TAATACGACTCACTATAGGG
	R	TAGTTATTGCTCAGCGGTGG
pIB/V5-HIS TOPO® (OpIE2)	F	CGCAACGATCTGGTAAACAC
	R	GACAATACAAACTAAGATTTAGTCAG
TOPO® TA (M13)	F	GTAAAACGACGGCCA
	R	CAGGAAACAGCTATGAC

[a] All primers were ordered from Gibco.

[b] Primers for expression in prokaryotic expression vector (pET/100 Directional TOPO®).

[b] Primers for expression in eukaryotic expression vector (pIB/V5-HIS TOPO®).

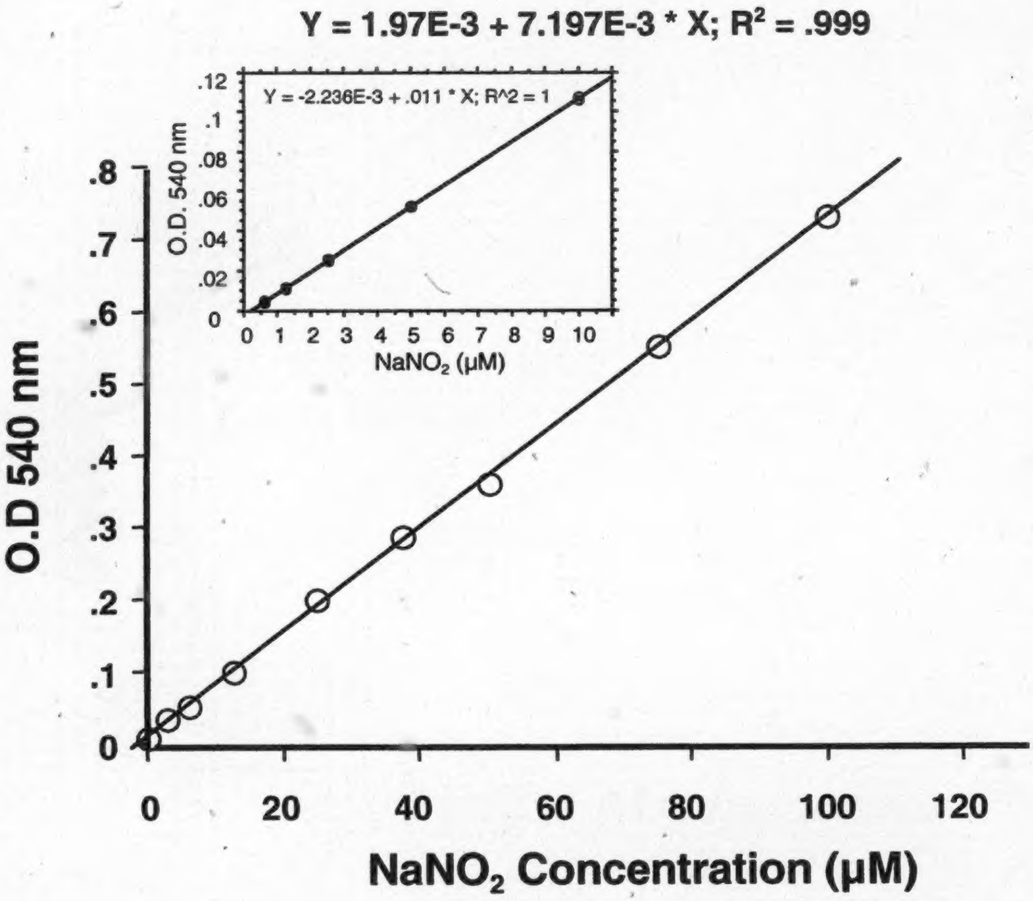

Figure 1.1. Sodium nitrite standard curve for the determination of NO production using the Griess reaction. Inset: Sodium nitrite standard curve for low values of nitrite (i.e. 1.0-10 µM).

CHAPTER 3

IN VITRO CULTIVATION OF FISH MACROPHAGES[2]

3.1 Introduction

Macrophages are immune cells that are found in all metazoan organisms, ranging from primitive animals (e.g. starfish) to humans. These cells act as the first-line of innate defense by recognizing and eliminating foreign invaders, and in higher organisms participate in the initiation of the acquired immune responses. In mammals, macrophages originate from blood monocytes, which in turn originate from progenitor cells in the bone marrow. Macrophage sub-populations are ubiquitously distributed throughout the tissues of the body according to the mononuclear phagocyte system theory [532]. These cells are terminally differentiated and have limited proliferative abilities. Tissue macrophage populations are replenished by the infiltration of monocytes that undergo site-specific maturation in response to a variety of tissue 'cues'. However, there is also evidence that macrophages can undergo self-renewal and are not necessarily dependent on the monocyte infiltration for maintenance of their numbers. For example, fetal liver macrophages appear prior to the formation of the bone marrow [533-534] and microglia (i.e. brain macrophages) are at a site that is not accessible to infiltrating monocytes and appear to be regenerated by microglia-specific progenitors [535]. Furthermore, elimination of circulating monocytes by irradiation, does not affect the total numbers of resident macrophage populations in the peritoneum, lungs or liver [536-538]. These findings suggest that there are resident populations of tissue macrophages that are capable of self-renewal and are not derived from bone marrow progenitors and circulating monocytes.

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[2] A version of this chapter has been published: Stafford *et al.*, 2001. *Dev. Comp. Immunol.*, 25: 447-459; and Joerink *et al.*, 2003. *SEB Exp. Biol. Reviews*. 55th Edition (in press).

Macrophages proliferate in response to exogenous macrophage colony stimulating factor (M-CSF or CSF-1) [539-542] and other growth factors such as IL-6, IL-3 and GM-CSF [543-545]. In mammals, M-CSF is the most important cytokine involved in the proliferation of macrophages that acts on all macrophage progenitor cells, directing cell proliferation towards monocyte development. Unlike mammals, the precise events mediating fish macrophage growth and proliferation are poorly understood and to date the native factors that mediate these events have not been identified. However, fish macrophages appear to produce endogenous growth factors that can act in an autocrine manner to regulate their own growth and differentiation [321, 464, 546]. This process has not been reported to occur in mammals.

The ability to isolate and culture fish macrophages over extended periods of time is of paramount importance for not only understanding the events of fish macrophage hematopoiesis but also for the generation of functional immune cells. Different labs have successfully established long-term macrophage cultures from various fish species [464, 547-551]. Recently, we developed a culture system that obtained high yields of macrophages from the hematopoietic tissue (head kidney) of goldfish (*Carassius auratus*) [321, 336, 464]. This method involved the isolation of kidney leukocytes using a 51% Percoll gradient, and incubation of these cells in the presence of cell conditioned-medium (CCM). The CCMs contained macrophage growth factors (MGFs), which were secreted by both goldfish kidney leukocytes and a goldfish macrophage cell line (GMCL) [321, 464]. The endogenously produced

MGFs were also shown to support proliferation and differentiation of primary kidney-derived macrophages [336]. After 8-12 days of cultivation in the presence of CCM, freshly isolated goldfish kidney leukocytes developed into *in vitro* derived kidney macrophages (IVDKM) cultures. Flow cytometric analysis indicated that these cultures are composed of three distinct macrophage sub-populations, designated R1, R2, and R3 [321, 335-336].

Goldfish R2 cells are morphologically and cytochemically similar to mature tissue macrophages and produce both reactive oxygen (ROI) and reactive nitrogen intermediates (RNI) in response to immunological stimuli [318-319, 322, 336]. The R3 cells are morphologically and cytochemically similar to monocytes and produce both ROI and RNI in response to immunological stimuli. However, goldfish monocyte-like cells produce very little RNI, similar to mammalian monocytes [336]. The R1 or progenitor-like cells do not produce ROI or RNI and appear to be important for the development of IVDKM and generation of the R2 and R3 sub-populations [335-336, 552].

This chapter described the adaptation of goldfish macrophage cultivation procedures for the isolation and cultivation of cells from two other economically important fish species, the common carp (*Cyprinius carpio*) and rainbow trout (*Oncorhynchus mykiss*). To date, relatively little work has been done on the cultivation of primary macrophage cultures from these species. Specifically, I will show that the techniques used to generate goldfish primary macrophage cultures can be successfully adapted to obtain macrophage-like cells from carp (*Cyprinius carpio*) and monocyte-like cultures from rainbow trout (*Oncorhynchus mykiss*). The main objectives of the experiments reported in this chapter are: 1) to generate and characterize primary macrophage cultures from another cyprinid species (i.e. carp) and show that these cultures contain cells that are similar to goldfish IVDKM based on flow cytometry, function, morphology and cytochemical staining; 2) to show that the cultivation of rainbow trout head kidney leukocytes, in the presence of CCM enriched for functional monocyte-like cells; and 3) to characterize trout primary kidney monocytes (T-PKM) based on their response to CCMs, flow cytometric profiles, microscopic analysis, myeloperoxidase staining, and generation of antimicrobial products.

3.2 Results

3.2.1 Flow cytometric analysis of primary cultures derived from different fish species

Figure 3.1 shows a typical flow cytometric profile of kidney-derived macrophage cultures from goldfish, carp and rainbow trout. Goldfish macrophage cultures consisted of three major sub-populations designated as R1, R2, and R3. Morphologically, cytochemically, and functionally these cells represented early progenitor- (R1), monocyte- (R3), and macrophage-like cells (R2) [321, 336]. Based on flow cytometry, carp cultures were comparable to goldfish cultures and also consisted of three major sub-populations. However, cultures derived from the rainbow trout had a significantly different flow cytometric profile although they also contained three identifiable sub-populations.

3.2.2 Analysis of *in vitro*-derived kidney macrophages from carp

Carp kidney leukocytes cultured in the presence of 25% CCM exhibited the development of three sub-populations over increasing incubation periods (Fig 3.2). These results are similar to the development of goldfish IVDKM, a closely related cyprinid species. Between days 6 and 8, three sub-populations of cells were clearly detectable by flow cytometry. However, after 10 days of cultivation, the cells appeared to enter into a phase of senescence based on the flow profiles, a similar effect was also observed in goldfish cultures [552]. Microscopic analysis revealed that carp sub-populations were morphologically indistinguishable from their goldfish counterparts with the R2 cells being macrophage-like and the R3 cells being monocyte-like. Carp R2 cells were large and irregularly shaped (12-20 μm in diameter) with a low nucleus to cytoplasm ratio and often had extensive membrane ruffling and vacuolization [553]. These cells stained positive for esterase (α-naphthyl acetate esterase) and acid phosphatase but were negative for myloperoxidase. Carp R3 cells were large round cells (12-15 μm in diameter) with a low nucleus to cytoplasm ratio and often had eccentrically placed nuclei. The R3 cells stained positive for acid phosphatase but negative for both esterase and myeloperoxidase activity [553].

Functionally, both R2 and R3 cells were capable of producing NO after appropriate stimulation (Fig. 3.3). However, the sorted macrophage-like cells produced higher amounts of NO than the sorted monocyte-like cells, which was similar to that observed for goldfish [336].

3.2.3 Cultivation of rainbow trout head kidney leukocytes and the development of trout-primary kidney monocyte-like cultures (T-PKM)

Trout head kidney leukocytes cultured in the presence of trout CCM were monitored over-time by flow cytometry (Fig. 3.4). This approach resulted in the identification of three sub-populations of cells based on forward- and side-scatter parameters (FSC and SSC). These sub-populations were gated according to foci of cells that were identified using density plot analysis and were designated as R1, R2, and R3 (Fig. 3.4; trout flow cytometry template). At least 20 individual trout kidney leukocyte preparations were examined, and in all cases these three sub-populations of cells were observed. The differences between goldfish or carp IVDKM and the T-PKM flow cytometry gates represents the differences in size and internal complexity of cultured kidney leukocytes from these different fish species.

An example of the changes in flow cytometric profiles of trout leukocytes over extended cultivation periods is shown in Fig. 3.4. This representative trout leukocyte culture demonstrated the standard flow cytometric gating pattern observed after cultivation of the cells in the presence of 25% CCM. In all fish examined, the %R3 population increased after extended cultivation (i.e. 8-12 days), while the other sub-populations did not. Initially, after 4 days of cultivation, the majority of cells gated to the R1 and R2 regions. Subsequent cultivation demonstrated a steady decline of the R1-gated cells, no change in the R2-gated cells, and a gradual increase in the R3-gated cells. The trend for this consistent enrichment of the R3 sub-population was observed for all T-PKM cultures examined and the results are summarized in Table 3.1.

3.2.4 Identification of an R3-enriching factor in T-PKM-derived CCM

To confirm the presence of an R3-enriching factor within trout CCMs, trout leukocytes were cultured in the presence of complete medium alone (no CCM) or were cultured in complete medium supplemented with 25% (v/v) CCM. Two separate CCMs were tested (designated CCM1 and CCM2). T-PKM cultures were subsequently analyzed after 4, 8, and 12 days, using flow cytometry. The cultivation of T-PKM in the presence of two different CCMs resulted in an enrichment of the R3- cell sub-population during the first 12 days of cultivation (Fig. 3.5), an effect that was not observed when cells were cultured in medium alone (i.e. without CCM).

3.2.5 Morphological and cytochemical characterization of T-PKM cultures

The R3 cells from T-PKM cultures were subsequently sorted using the flow cytometer and cytospin smears were made of pre-sort and sorted cells (Fig. 3.6). The sorted R3 cells were large round cells that often had eccentrically placed kidney-shaped nuclei (Fig. 3.6B). In addition, these cells demonstrated dark and diffuse staining for myeloperoxidase (Fig. 3.6B1). Based on these morphological and cytochemical characteristics, the R3 sub-population was classified as monocyte-like cells. These monocyte-like cells were also the largest cells within T-PKM based on FSC, SSC, and microscopic analysis, ranging in size from 10-15 µm. Morphologically, the other cell types in T-PKM (pre-sort) were small, round, and contained very little cytoplasm (similar to R1 cells of goldfish IVDKM), or resembled lymphocytes with large round nuclei and very little cytoplasm (R2 cells) (Fig. 3.6A). Some of the cells in the R2 region may be promonocytes due to their size and dark-blue staining cytoplasm, which is a characteristic of some immature cell types. Interestingly, a mature macrophage-like sub-population in T-PKM was not observed. In contrast, goldfish and carp IVDKM cultures were composed of cells with typical monocyte-like morphology (i.e. round cells with kidney-shaped nuclei) and macrophage-like morphology (i.e. large irregularly shaped cells with extensive vacuolisation). Flow cytometric analysis of T-PKM cultures failed to demonstrate the presence of a mature macrophage-like cell population (Fig. 3.6A).

3.2.6 Identification of functional (ROI-producing) sub-populations in primary fish cultures (goldfish IVDKM and T-PKM)

3.2.6.1 Goldfish IVDKM

The production of ROI by goldfish macrophages was assessed using the fluorescent probe dihydrorhodamine (DHR) using a modified procedure for the analysis of mammalian neutrophil respiratory burst activity [529-531]. Goldfish kidney macrophages were loaded with 10 μM DHR and the respiratory burst was triggered by the addition of 100 ng PMA. Cells not exposed to PMA were used to establish the base-line fluorescence intensity (FL1) (approximately 10^1 on the log scale). As shown in Figure 3.7B, IVDKM cultures loaded with DHR demonstrated a pronounced peak on the FL1 histogram plot. After triggering with PMA, there was a shift in the fluorescence intensity (Fig. 3.7B) due to the release of respiratory burst products. DHR staining and flow cytometry allowed for the analysis of the respiratory burst activity of different sub-populations within the heterogeneous goldfish IVDKM cultures. The sub-populations responsible for the oxidation of DHR to Rho 123 (i.e. shift in fluorescence intensity) were identified as the R2- and R3-type cells (Fig

3.7C). The R1-type cells did not have a detectable respiratory burst response. The respiratory burst activity was expressed as mean fluorescence intensity per cell in the individual sub-populations. For all goldfish cultures examined, the monocyte-like (R3) cells had a significantly higher respiratory burst activity when compared to the macrophage-like (R2) cells ($P<0.05$).

3.2.6.2 T-PKM

DHR and flow cytometry were also used to identify the functional population of ROI-producing cells within T-PKM cultures (Fig. 3.8). Day 8 T-PKM cultures were used for analysis (Fig. 3.8A). Triggering with 100 ng PMA caused a pronounced shift in the fluorescence histogram (Fig. 3.8B) indicative of the presence of respiratory burst products. In contrast to goldfish macrophage cultures, only one population of cells appeared to produce ROI. The population of cells responsible for this shift were identified as the R3 sub-population (i.e. monocyte-like cells; Fig 3.8C). Interestingly, this was the same population that survived and appeared to proliferate during extensive culture periods (i.e. 8-12 days) in the presence of 25% CCM.

3.2.7 Production of RNI by goldfish IVDKM and T-PKM

Stimulation of 8-12 day old goldfish IVDKM and T-PKM demonstrated that only IVDKM were capable of producing RNI following immunological stimuli (Fig. 3.9). Flow cytometric analysis of goldfish IVDKM and T-PKM, using identical instrument settings, clearly demonstrated that the T-PKM cultures did not have a sub-population of cells equivalent to mature goldfish macrophages (Fig. 3.9). The ROI-producing sub-population in T-PKM cultures gated more closely to the monocyte-like cells found in goldfish IVDKM.

3.3 Discussion

The cultivation of goldfish kidney leukocytes in the presence of CCM, derived from previously established goldfish macrophage cultures, resulted in the generation of IVDKM [321, 336, 496]. Goldfish IVDKM cultures also contained three distinct sub-populations of cells. These sub-populations appear to represent cells that are developmentally arrested at different stages of macrophage differentiation (i.e. early progenitors, monocytes, and macrophages, designated R1, R3 and R2, respectively) [335-336, 552]. Based on the experiments presented in this chapter, the cultivation of carp kidney leukocytes in the presence of CCM also resulted in the generation of IVDKM cultures. These cultures were practically indistinguishable from goldfish IVDKM based on morphological, cytochemical, flow cytometric, and functional analysis. This is not surprising, however, since carp is closely related to goldfish. To date, most studies examining the immunological functions of carp macrophages have relied on density gradient centrifugation resulting in leukocyte fractions ‘enriched’ for macrophage-like cells. Subsequent adherence to plastic was commonly performed for a final enrichment of up to 60% macrophages [554]. However, the presence of neutrophilic granulocytes comprises a major impurity, and thus hinders a definitive conclusion on macrophage functions, and for this reason many studies using carp cautiously refer to ‘phagocytes’ [553]. My results showed that the adaptation of the goldfish cultivation procedures for the development of carp IVDKM was possible and thus may enable more precise studies on these highly enriched cultures.

In contrast, my results showed that the cultivation of rainbow trout head kidney leukocytes, in the presence of CCM, generated only a single functional population of cells, which appeared to be monocyte-like. The increased %R3 (monocyte-like cells) during extended cultivation periods, in the presence of CCM, suggests that there is at least one MGF present in trout-derived CCM. The majority of smaller non-functional cells present in early cultures of T-PKM are most likely lymphocyte-like cells, which has been previously reported [555]. However, some of these cells may also represent pro-monocyte-like cells (i.e. R2 in T-PKM), which in the presence of appropriate factors found in CCM, differentiate into the monocyte-like cells. Therefore, within the hematopoietic tissue of the trout kidney, monocytes differentiate from pro-monocytes and these monocytes likely enter the circulation and eventually become tissue macrophages. This pathway of differentiation is similar to that of mammalian macrophages. In contrast, goldfish and carp macrophages are capable of differentiating fully *in vitro*. Detailed analysis of the differentiation and proliferation events that occur during the development of goldfish IVDKM cultures revealed that: a) these cultures develop in three phase of growth (i.e. lag-, proliferative-, and senescence-phases); b) the mature macrophage-like subpopulations develop from both, the differentiation of monocyte-like cells, and direct differentiation of early progenitors; and c) mature goldfish macrophage-like cells appear to be capable of self-proliferation/renewal [321, 334-336, 552].

T-PKM also exhibited different functional properties when compared to goldfish or carp IVDKM. Goldfish and carp IVDKM cultures contained a sub-population of cells with the ability to generate large amounts of RNI in response to immunological stimuli. This is one of the functional hallmarks of mature goldfish macrophages [336]. It appears that one of the necessary events for NO production by goldfish IVDKM involves an increased differentiation of monocyte-like cells into mature macrophage-like cells, which is similar to the differentiation pathway observed in mammals [185-186, 493-494, 510, 556]. In contrast, T-PKM cultures did not produce RNI but were capable of generating ROI as demonstrated using the DHR assay. A single monocyte-like sub-population was responsible for the ROI response in T-PKM cultures, while two ROI-producing sub-populations were present in goldfish IVDKM (i.e. monocyte- and macrophage-like cells).

In mammals, cultured monocytes undergo a variety of changes that may be similar to those that occur after migration into the tissue from the peripheral blood [557-559]. In addition, cultured human monocytes are believed to resemble human resident tissue macrophages [558-559]. Therefore, cultivation of monocytes over several days resulted in their differentiation and a more mature macrophage phenotype. The differentiation of the monocytes in culture was concomitant with changes in the generation of both ROI and RNI [560]. Freshly isolated human monocytes have a high capacity to generate ROI, and this response was shown to decline after 4 days of cultivation [560]. Conversely, the production of RNI was not detectable in freshly isolated monocytes but after 4 to 5 days of cultivation, they acquired the ability to produce RNI [560]. A similar process may be important for the induction of the RNI response by goldfish macrophages. Goldfish monocyte-like cells produce very little RNI when compared to the mature macrophage-like cells [336]. It has also been demonstrated that goldfish monocytes could be induced to differentiate into macrophage-like cells in response to activating stimuli (i.e. MAF or LPS) as well as CCM [336]. This differentiation step may be a necessary event for RNI production by goldfish macrophages. In contrast, the inability of T-PKM cells to produce RNI may be due to an absence of a mature macrophage-like sub-population in the cultures. The reasons for the absence of this population in T-PKM cultures are not known, but may involve the absence of necessary factor(s) required for the functional differentiation of monocytes into mature macrophages *in vitro*. My results showed that prolonged cultivation of trout leukocytes in the presence of CCM failed to induce the differentiation of the monocyte-like cells into mature macrophages capable of producing RNI. Attempts to induce this differentiation step with LPS were also unsuccessful (data not shown). Recently, others have used the cultivation techniques described in this chapter to obtain monocyte-like cultures from rainbow trout [341]. Moreover, this group attempted to induce the differentiation of these monocytes into macrophages. While the 'differentiated' cells exhibited an increase in phagocytic capacities and expression of TNFα, there was no conclusive evidence presented that these cells are indeed *in vitro*-differentiated trout macrophages. In addition no cytochemical staining was performed and the production of RNI by these cells was not reported. Thus, further work is required to obtain fully functional *in vitro*-derived trout macrophages.

During an inflammatory response in trout, kidney-derived monocytes are recruited to the inflammatory site [466] and, in tissues, presumably differentiate into mature macrophages that can produce RNI, as iNOS expression has been detected in trout kidney [561]. However, the ability of kidney leukocytes to produce RNI appears to be rapidly down-regulated *in vitro* [467]. Thus, trout kidney macrophages and T-PKM cultures, unlike goldfish macrophages, may require the continuous presence of other appropriate tissue 'cues' for subsequent differentiation into fully functional mature macrophages, with the ability to produce RNI.

In conclusion, the results presented in this chapter demonstrated that enriched primary monocyte-like cultures could be derived from trout head kidney leukocytes, using techniques developed for the establishment of goldfish and carp IVDKM. However, these cells require as yet unknown signals to differentiate into fully mature macrophages with the ability to produce RNI. The enrichment for a functional sub-population of monocyte-like cells by the addition of homologous CCM, suggests that there is at least one growth factor present in trout CCM, which is yet to be identified. The use of these cultivation techniques will be invaluable for the enrichment of functional monocyte- and macrophage-like cells from cyrinid and salmonid fish species. These cells can subsequently be used in bioassays that measure cell-mediated immune responses of fish exposed to a variety of agents (i.e. environmental contaminants, pathogens, or immunization) and for the further characterization of the different MGFs responsible for fish monocyte/macrophage development.

Table 3.1. Proportion of R1, R2, and R3-type cells in trout primary monocyte like cultures[a]

	Cells (Total: 10 000 events collected)							
	Fish 1	**Fish 2**	**Fish 3**	**Fish 4**	**Fish 5**	**Fish 6**	**Mean**	**Range**
R1	22.9%	23.8%	5.6%	20.9%	8.1%	6.8%	14.6	5.6-23.8%
R2	5.8%	8.4%	4.3%	2.7%	5.1%	6.8%	5.5	2.7-8.4%
R3	42.9%	50.1%	77.5%	33.7%	55.9%	62.6%	53.8	33.7-77.5%

[a] Trout head kidney leukocytes were isolated using a 51% Percoll gradient and the viable leukocytes seeded into 25 cm^2 ($10x10^6$/flask) flasks in complete growth medium supplemented with 25% (v/v) CCM. Using the standard gates derived from analysis of T-PKM cultures, the proportions of cells within each region was determined after 8-12 days in culture. This data demonstrated the enrichment of the R3 sub-population after cultivation of trout leukocytes in the presence of 25% CCM.

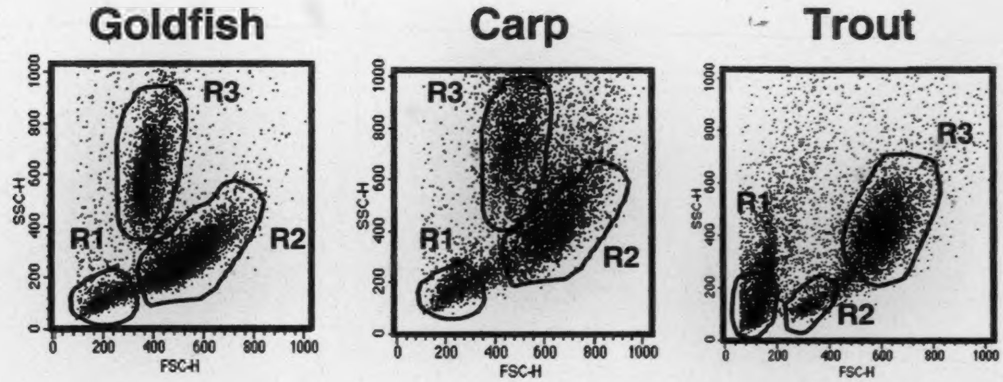

Figure 3.1. Flow cytometric analysis of *in vitro*-derived kidney macrophage cultures from goldfish, carp, and rainbow trout. Kidney leukocytes were isolated from individual fish and cultured in the presence of 25 % (v/v) homologues CCM. After, 8-12 days incubation at 20°C, aliquots of cultures were removed and analyzed by flow cytometery. Results are from individual fish cultures and are representative of a minimum of 10 primary macrophage cultures that were developed from each species.

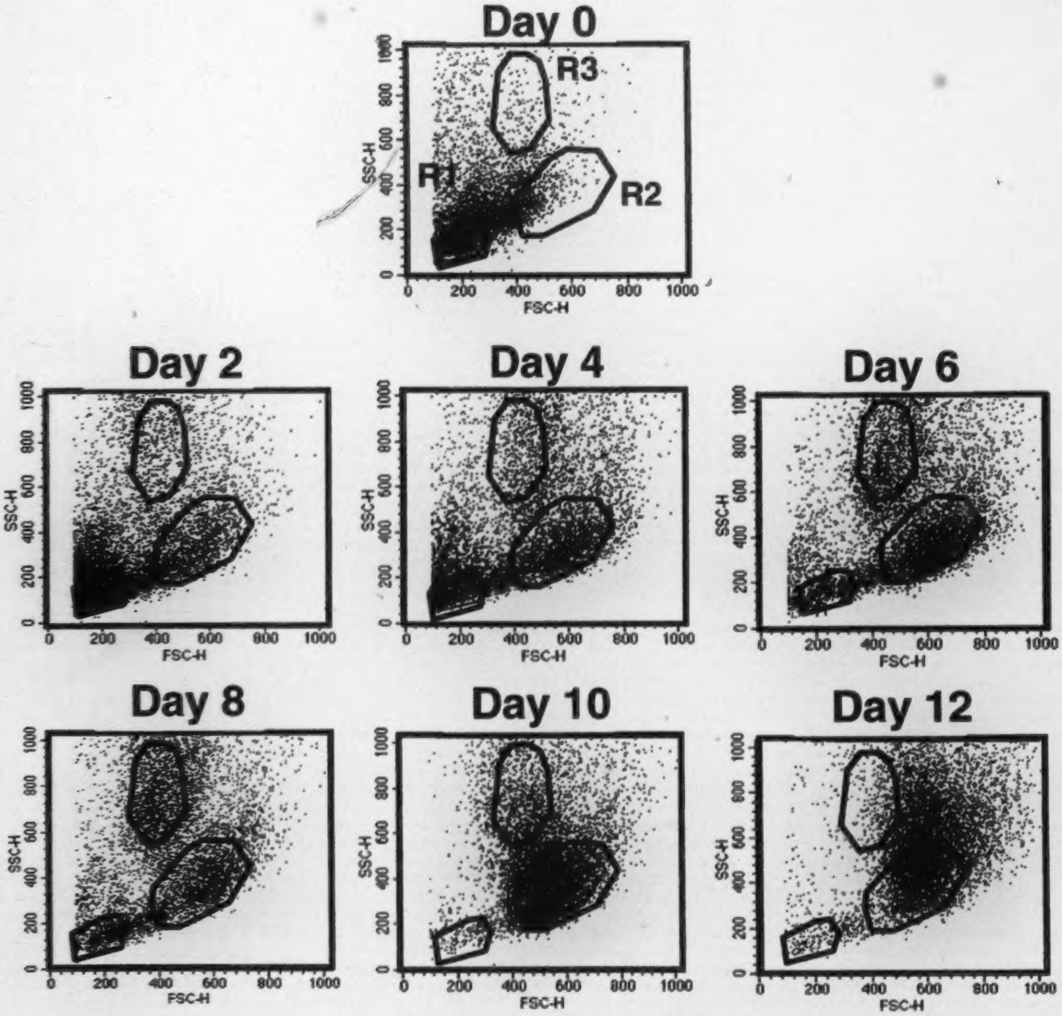

Figure 3.2. Cultivation of carp kidney leukocytes and the generation of carp IVDKM cultures. Carp kidney leukocytes were isolated using 51% Percoll and seeded into 25 cm^2 (10x10^6 /flask) flasks in complete growth medium supplemented with 25% (v/v) CCM. Sub-samples were removed from the culture flasks on the indicated days and analyzed by flow cytometry. Data is from a representative fish out of 20 different carp cultures that were generated.

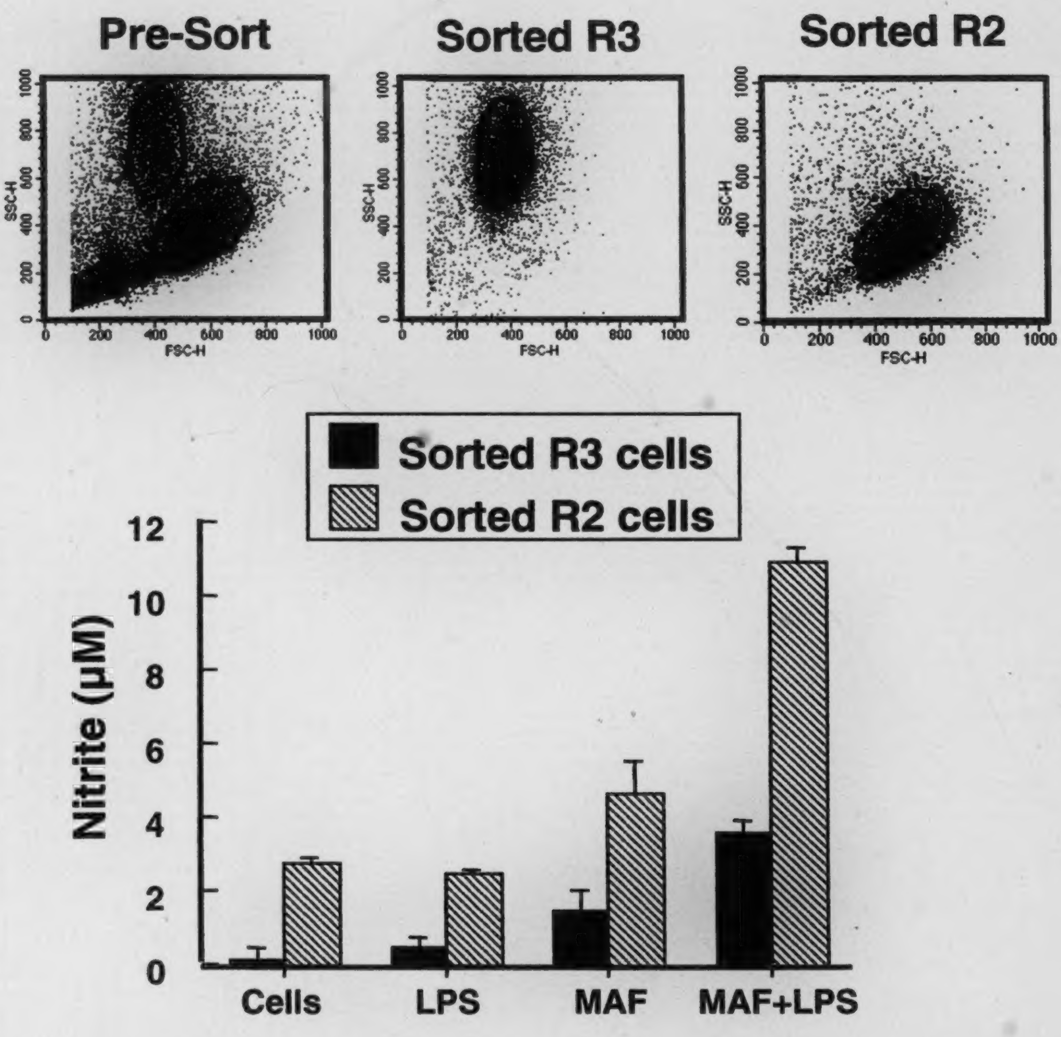

Figure 3.3. Production of NO by sorted sub-populations from carp IVDKM cultures. A pre-sort profile of 10 pooled day 8 cultures was used to establish the sort gates. Sorted cells were then seeded into 96-well culture plates (5×10^4 cells/well) and treated with medium (control), 10 µg/ml LPS, MAF (1:4 final dilution), or a combination of MAF and LPS. After 72 h incubation at 20°C, NO production was determined using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and the data is from a representative experiment out of three independent experiments that were performed.

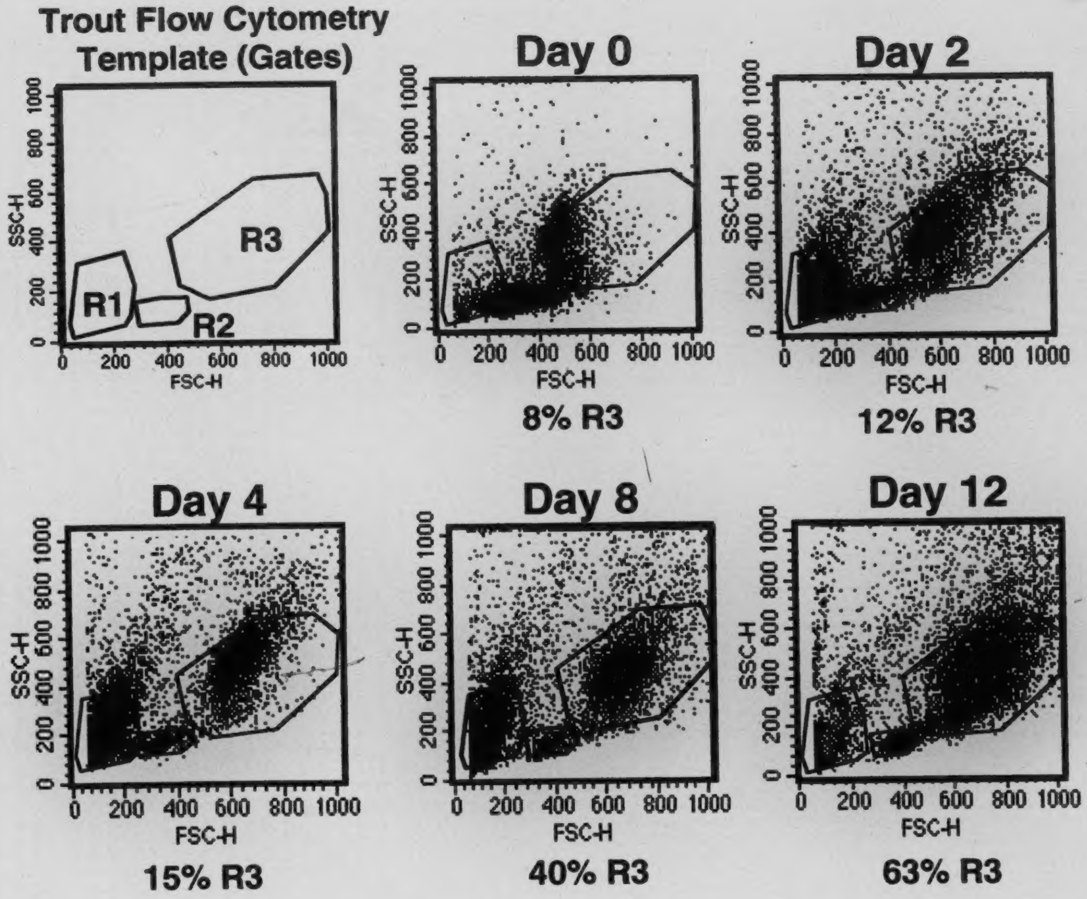

Figure 3.4. Cultivation of trout head kidney leukocytes and the generation of trout T-PKM cultures. Rainbow trout head kidney leukocytes were isolated using 51% Percoll and seeded into 25 cm^2 ($10x10^6$ /flask) flasks in complete growth medium supplemented with 25% (v/v) CCM. Sub-samples were removed from the culture flasks on the indicated days and analyzed by flow cytometry. Data is from a representative fish out of 20 different rainbow trout cultures that were generated.

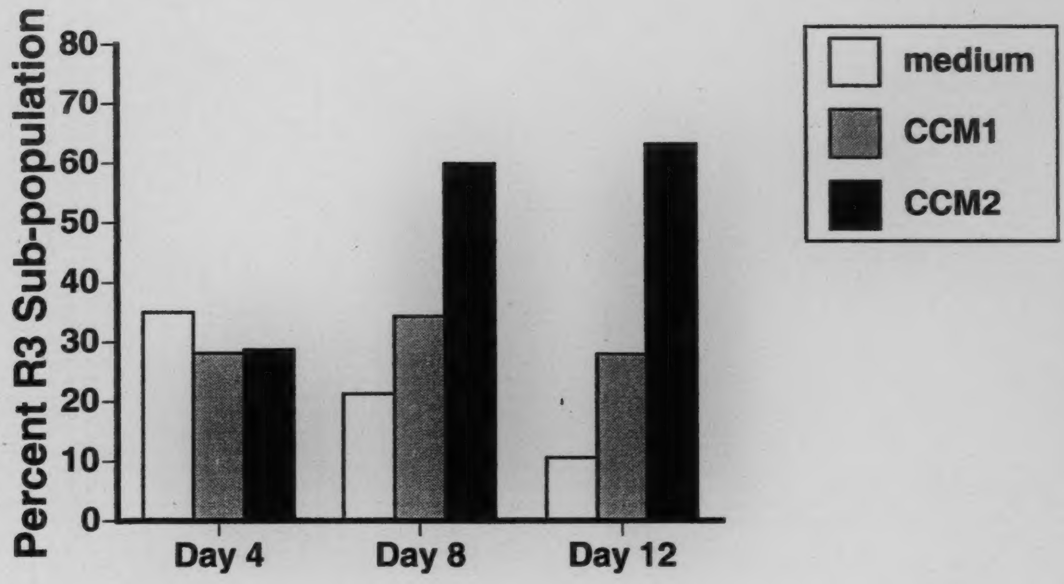

Fig. 3.5 Demonstration of an R3-enriching factor present in rainbow trout-derived CCM. Rainbow trout head kidney leukocytes were isolated using 51% Percoll and seeded into 25 cm^2 (10x10^6 /flask) flasks in complete growth medium supplemented with 25% (v/v) CCM. On days 4, 8, and 12, sub-samples were removed from the culture flasks and examined by flow cytometry. Using the trout flow cytometer template, the number of gated R3 cells was determined out of 10 000 total collected events. Two different CCMs were tested for R3-enriching activity (designated CCM 1 and CCM 2). Data is from a representative experiment out of two independent experiments performed.

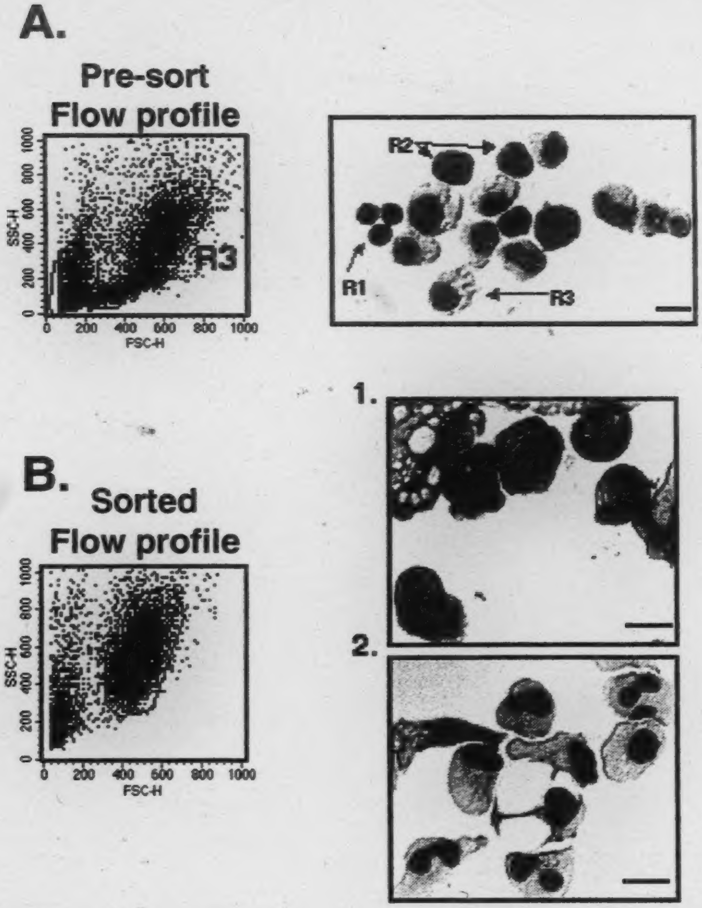

Fig. 3.6. Flow cytometric analysis of sorted R3-type cells from rainbow trout head kidney leukocytes after 8 days of cultivation in the presence of CCM. The R3-type cells were sorted using the flow cytometer. Three predominant cell types were observed in the pre-sort profile (R1, R2, and R3) (A). The R1 cells were the smallest and had a high nucleus:cytoplasm ratio (Scale bar 10µm). R2 cells were medium in size and were typically round cells with large round nuclei and blue-staining cytoplasm. R3 cells were round with kidney-shaped shaped nuclei. These cells displayed characteristic monocyte-like morphology and were the cell type identified following flow cytometric sorting (B). These cells were also positive for myeloperoxidase (Sigma) and demonstrated a dark diffuse staining pattern (B1).

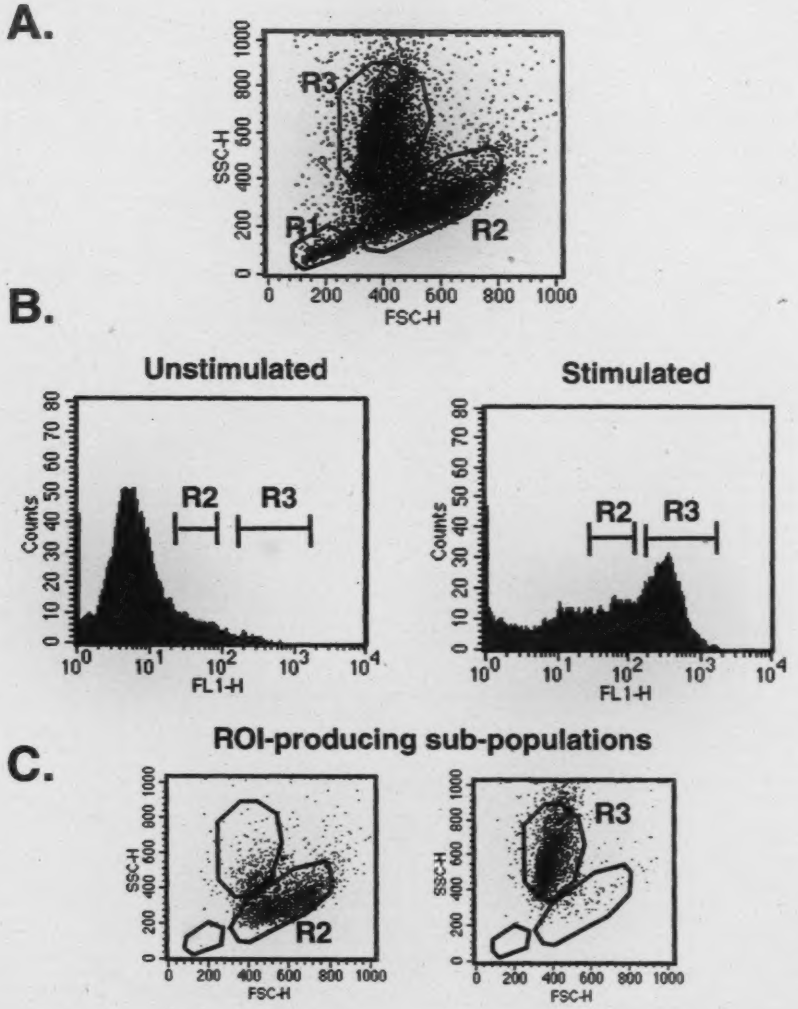

Fig. 3.7. DHR analysis of goldfish macrophage sub-populations found within goldfish IVDKM cultures. A typical 8-12 day old goldfish macrophage culture (A) was loaded with DHR (B). Unstimulated cells exhibited a fluorescence intensity (FL1) of less than $10^{1.5}$ (no ROI production). After PMA treatment, stimulated cells produced ROI, as indicated by a significant increase in fluorescence intensity (B). Region analysis identified two functional ROI-producing sub-populations (R2 and R3) in goldfish IVDKM based on an increase in FL1 fluorescence.

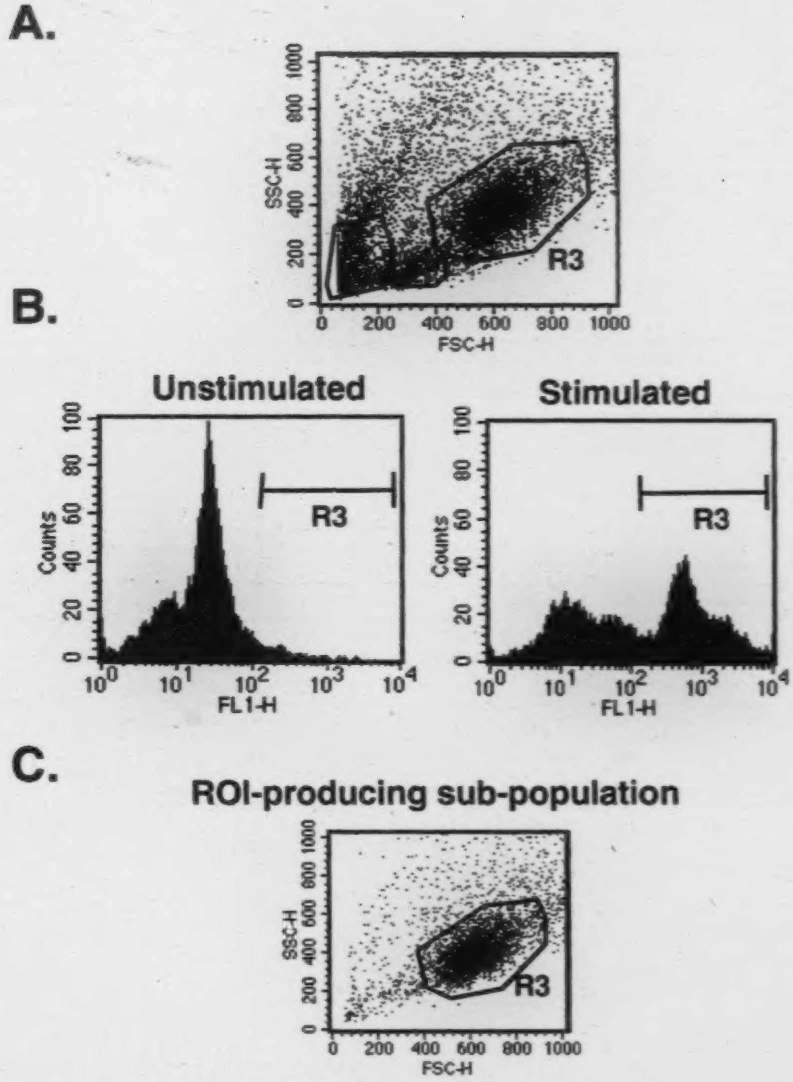

Fig. 3.8. DHR analysis of trout macrophage sub-populations found in T-PKM cultures. A typical 8-12 day old T-PKM culture (A) was loaded with DHR (B). Unstimulated cells exhibited a fluorescence intensity (FL1) of less than $10^{1.5}$ (no ROI production). After PMA treatment, stimulated cells produced ROI, as indicated by a significant increase in fluorescence intensity (B). Region analysis identified one functional ROI-producing sub-population (R3) in trout cultures.

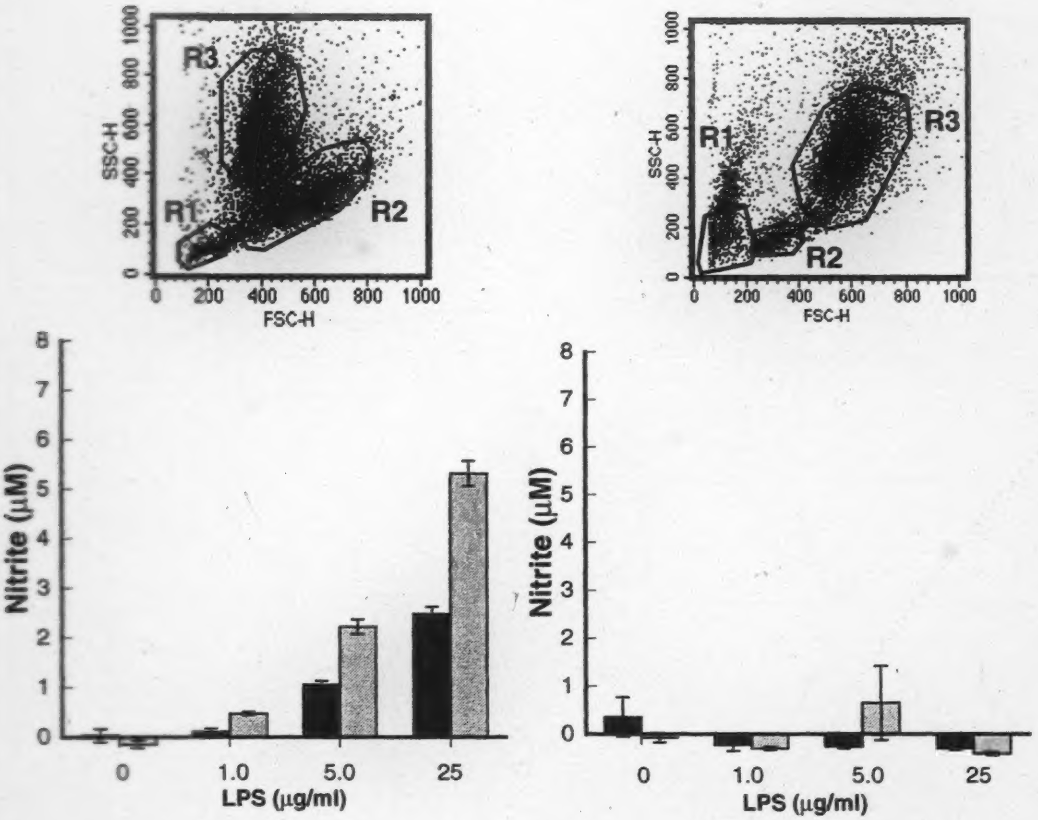

Fig. 3.9. Nitric oxide (NO) production by goldfish IVDKM and T-PKM. Goldfish and trout kidney leukocytes were cultured in the presence of 25% (v/v) homologues CCM and after 8-12 days, the cells were harvested and seeded into 96-well half area tissue culture plates ($5x10^4$ cells/well). Cells were stimulated with LPS (1 to 25 μg/ml; black bars) or homologues MAFs (1:4 final concentration; shaded bars) and LPS. Cells were then incubated for 72 h at 20°C prior to determination of the NO response. Each bar represents the mean ± SEM of triplicate cultures and the data are from a representative experiment out of three independent experiments that were performed.

CHAPTER 4

INHIBITION OF GOLDFISH MACROPHAGE ANTIMICROBIAL FUNCTIONS BY ENDOGENOUS AND EXOGENOUS FACTORS[3]

4.1 Introduction

Teleost macrophages become activated following stimulation with exogenous factors (i.e. bacterial LPS) and/or soluble host-derived factors [314, 317-318]. The activation of fish macrophages can subsequently be monitored by detecting the up-regulation of various genes (e.g. iNOS) and/or by measuring the release of antimicrobial molecules (i.e. ROI and RNI) [318, 322, 337, 562]. We have recently demonstrated that mitogen-stimulated goldfish kidney leukocytes secrete several factors with immunomodulatory properties [322]. These endogenous factors were shown to induce the production of ROI and RNI in activated goldfish macrophages and preliminary biochemical and functional characterization of both RNI and ROI-inducing factors has been performed [322]. However, we know very little about the identification of the native factors that mediate these responses.

In contrast, significantly more information is available regarding the native factors that modulate mammalian macrophage functions. For example, cytokines responsible for the induction of RNI and ROI production in mammalian macrophages have been extensively characterized and include IFNγ, GM-CSF, TNFα, and IL-2 (Reviewed by [563-564]). Several cytokines have also been shown to inhibit macrophage activation and subsequent production of NO and include TGFβ [565], IL-4 [566], and IL-10 [567]. Non-cytokine molecules such as steroids also exhibited potent macrophage inhibitory properties (i.e. prostaglandin E_2 [568]). Since the production of NO can potentially damage host cells and proteins in close proximity to activated macrophages, the generation of this molecule is under tight regulatory control [411]. The regulation and coordination of macrophage-mediated immune response is achieved by balancing the production of pro- and anti-inflammatory mediators [564].

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[3] A version of this chapter has been published: Stafford *et al.*, 1999. *Dev. Comp. Immunol.*, 23: 585-596; and Stafford *et al.*, 2002. *Dev. Comp. Immunol.*, 26: 445-459.

In addition to endogenous factors (i.e. cytokines), we have recently demonstrated that potassium channel activity mediated macrophage antimicrobial functions. Specifically, pharmological blockers of potassium channels were shown to significantly inhibit the production of NO by activated murine macrophages [569] [570]. Others have also demonstrated that potassium channel activity is important for regulating immune cell functions [571-578]. Until recently, the roles of potassium channels in mediating fish macrophage antimicrobial responses had not been addressed [579].

To date, the majority of native fish macrophage modulating factors have been demonstrated in crude supernatants derived from mitogen-stimulated fish leukocytes. From these crude supernatants, cytokine-like activities resembling mammalian IFNα, IL-1, IL-2, and TGFβ have been reported [323, 580-588]. In addition, the recombinant mammalian cytokines, TGFβ and TNFα, appear to functionally cross-react with teleost macrophages [327, 355]. Several fish cytokines have also recently been cloned and sequenced, and include IL-1β, TNFα, TGFβ, IL-6, and IFN [357-358, 367, 589-597]. These findings suggest that fish macrophage antimicrobial functions, like mammals, are regulated through the concerted actions of multiple host-derived factors. However, there are very few recombinant or native cytokines available from fish [363, 381] and as such, crude preparations are still extensively used for the modulation of fish macrophage antimicrobial responses [318, 322-323, 325].

Deactivation of activated mammalian macrophages and the subsequent inhibition of NO production has been well characterized and is mediated by several leukocyte-derived 'deactivating' cytokines such as TGFβ, IL-10, IL-4, and IL-13 [566-567, 598-599]. Inhibition of mammalian macrophage NO production by these factors was time- and dose-dependent and was a result of the modulation of iNOS gene and protein expression and/or stability [567, 598]. Few studies in fish have attempted to characterize the factors that exhibited macrophage deactivating activities [322-323, 328] and as such the main objectives of this chapter were to characterize the factors that inhibited goldfish macrophage antimicrobial functions. Specifically, the results presented in this chapter will describe the characterization of both endogenous (i.e. leukocyte-derived) and exogenous (i.e. pharmacological blockers of potassium channels) factors that significantly down-regulated the production of ROI and RNI in activated goldfish macrophages. Here I demonstrated that mitogen-stimulated goldfish kidney leukocyte supernatants contain at least one factor with potent inhibitory properties. The functional and biochemical characterization of this goldfish macropahge deactivation factor (MDF) will be described. My results will also show that in addition to an endogenous leukocyte-derived MDF, pharmacological blockers of potassium channels significantly downregulted goldfish macrophage antimicrobial functions. Until recently, the role of potassium channels in mediating fish macrophage antimicrobial responses had not been addressed. The results presented in this chapter demonstrate for the first time that the production of both ROI and RNI by goldfish macrophages was significantly impaired following pharmacological modulation of goldfish potassium channel activity, which was related to changes in plasma membrane potential (V_m).

4.2 Results

4.2.1 Generation of crude leukocyte supernatants by mitogen stimulation and MLR

To determine the relative contribution of MLR and mitogenic stimulation on the production of supernatants with MAF activities, goldfish kidney leukocytes were isolated from individual fish, treated with mitogens, and MAF activity of the supernatants were measured using the NO bioassay (Table 4.1). The mitogen-induced kidney leukocyte supernatants from 3 out of 5 individual fish induced NO production by IVDKM. Fractionation of these crude supernatants using GP-FPLC resulted in the identification of two major biological activities (i.e. MDF and MAF; Fig. 4.1). If the GP-FPLC fractions were incubated with IVDKM 6 h prior to stimulation with crude MAF and LPS, a significant NO-inhibitory activity was observed in some of the fractions (Fig. 4.1B). This MDF activity was maximally observed in GP-FPLC fraction #39, which was estimated to have an MW of 5-15 kD. However, if IVDKM were simultaneously incubated with the GP-FPLC fractions and LPS, NO-inducing activity was observed in the fraction with an approximate size of 50 kD (i.e. fraction #34; Fig. 4.1C). In some experiments, IVDKM treated with crude leukocyte supernatants produced very little or no NO and there was significant fish to fish variability of NO production in response to crude leukocyte supernatants (Fig. 4.2). The occasional inability of these crude supernatants to induce NO production may have been due to the presence of MDF(s). Therefore, fractionation of crude leukcoyte supernatants may segregate NO-inducing activity away from MDF activity, resulting in a more potent NO response in the partially purified samples (compare results in Tables 4.1 and 4.2).

4.2.2 Induction of NO production and iNOS gene expression

Similar to mammalian macrophages, activated goldfish macrophages exhibited an up-regulation of iNOS mRNA expression 6 h following stimulation, when compared to untreated controls (Fig. 4.3). Time-course analysis of NO production and iNOS mRNA expression demonstrated that significant levels of nitrite were not observed until 48h following stimulation (Fig. 4.4A). However, goldfish macrophage iNOS mRNA expression was increased as early as 2 h post-stimulation, peaked after 12 h and began to decline after 24 h (Fig. 4.4B).

4.2.3 Biochemical and functional characterization of an endogenous leukocyte-derived goldfish MDF

4.2.3.1 Reproducibility of MDF activity

To demonstrate that MDF activity could constantly be derived from mitogen-stimulated goldfish kidney leukocye supernatants, six individual crude leukocyte supernatants preparations were separated by GP-FPLC and all fractions tested for MDF activity. All fractionatated crude leukocyte supernatants demonstrated maximal MDF activity in fraction #39, with an observable variability in their NO-inhibiting capabilities (Fig. 4.5). The ability of this molecule(s) to antagonize the NO response of activated macrophages was not a result of macrophages dying after treatment with MDF, since similar numbers of viable macrophages were seen in cultures treated with the MDF-inducing fraction and PBS (Table 4.3).

4.2.3.2 Dose-response

MDF-mediated inhibition of NO production by stimulated goldfish macrophages was abrogated by serial dilution of the MDF, indicating a dose-dependency for MDF activity (Fig. 4.6). In addition, MDF antagonized the NO response of goldfish macrophages activated by an infection with the obligate intracellular parasite of mammalian macrophages, *Leishmania major*. This inhibition was also dependent on the dose of MDF used (Fig. 4.7).

4.2.3.3 Exposure of goldfish macrophages to MDF prior to activation significantly enhanced the NO-inhibitory effect

The GP-FPLC partially purified MDF induced approximately 70% inhibition of the macrophage NO response, provided that MDF was presented to the macrophage at least 6 h prior to activation with MAF and LPS (Table 4.4). If MDF was presented to the macrophage 24 h prior to activation, greater than 85% inhibition of NO production was observed (Table 4.4). Simultaneous treatment of macrophages with MDF in conjunction with MAF and LPS, resulted in only a 34% reduction in the NO response (Table 4.4). When the MDF was presented to the macrophages 24 h after the MAF and LPS signal, no significant differences were observed between NO production of control and MDF-treated macrophages indicating a time-dependancy of MDF-mediated inhibition of NO (Table 4.4).

4.2.3.4 Effect of incubation temperature on MDF activity

Incubation temperature also affected MDF activity. Inhibition of NO production by MDF after 24, 48, and 72 h were all statistically significantly ($P<0.0001$) when goldfish macrophages were incubated at 30°C (Fig. 4.8B). However, goldfish macrophages cultured at 20°C in the presence of MDF showed a significant inhibition of NO production only after 48 h ($P<0.009$) and 72 h ($P<0.0001$) incubations (Fig. 4.8A). This data suggests that inhibition of NO production was significantly higher and occurs earlier in goldfish macrophage cultures incubated at 30°C compared to those cultures incubated at 20°C.

4.2.3.5 C-FPLC (Mono-P) purification of MDF

GP-FPLC fractions containing maximal MDF activity (i.e. fraction #39) were subsequently concentrated approximately 60-fold using microcentrifugal concentrators with a 3 or 10 kD MWCO membrane. Most of the MDF activity was retained in the sample chamber of the concentrators indicating that the leukocyte-derived MDF(s) was larger than 10 kD (Fig. 4.9). Concentrated samples were then fractionated by C-FPLC using a Mono-P column. No MDF activity was observed in fractions collected during the descent of the pH gradient (Fig. 4.10). However, MDF activity was observed in the salt elution (SE), suggesting that the molecule(s) responsible for MDF activity was highly acidic with a $pI<4.0$ (Fig 4.11). The C-FPLC purified MDF-mediated inhibition of NO production by activated goldfish macrophages was similar to that of the GP-FPLC partially purified MDF (Fig 4.11).

SDS-PAGE demonstrated that GP-FPLC fractions containing maximal MDF activity contained at least three major protein bands with approximate MW of 12 , 15, and 16.5 kD (Fig. 4.12, lane 2). The 15 and 16.5 kD proteins did not appear to be responsible for MDF activity since they were not present in the C-FPLC SE fraction that contained MDF activity (Fig. 4.12, lane 3). Furthermore, subjecting partially purified MDF (i.e. GP-FPLC fraction #39) to C-FPLC, removed the 15 and 16.5 kD bands but retained a protein with an approximate MW 12 kD, indicating that the molecule(s) with MDF activity was 12 kD (Fig. 4.12, lane 3). This provided a more precise estimate of the size of the native MDF than compared with sizes estimated following Superose 6 fractionation (i.e. 5-15 kD).

4.2.4 Functional characterization of exogenous factors with goldfish macrophage deactivating activity

4.2.4.1 Potassium channel blockers inhibit production of NO in a dose-dependent manner

The potassium channel inhibitors 4-AP, quinine, and TEA caused a dose-dependent decrease in NO production of goldfish macrophages (Fig. 4.13). There were significant differences in the potency of NO inhibition by goldfish macrophages treated with the various potassium channel inhibitors. Quinine was the most potent inhibitor of NO production in goldfish macrophages with an IC_{50} of only 50 μM. In contrast, 4-AP and TEA were relatively weak inhibitors of NO production with IC_{50} values of 1.2 mM and 0.6 mM, respectively. At the highest doses tested, 4-AP, quinine, and TEA caused 80 %, 70%; and 50 % inhibitions of NO production by goldfish macrophages, respectively (Fig.4.13).

4.2.4.2 Potassium channel blockers significantly inhibit NO production by goldfish macrophages stimulated with LPS and/or crude MAF

Potassium channel blockers significantly inhibited NO production independent of the activation signal (i.e MAF and/or LPS). Goldfish macrophages activated with bacterial LPS, MAF, or MAF and LPS had significantly higher levels NO relative to untreated cells (Fig. 4.14). Treatment of activated macrophages with potassium channel blockers resulted in significant reductions in NO production (4-AP and quinine; P<0.0001) compared to controls. Cells treated with 10 mM TEA demonstrated significant reductions in NO production only following activation with LPS (10 μg/ml) (P<0.0001). Potassium channel blockers alone did not induce nitrite production when added to fish macrophage cultures (Fig. 4.14, cells alone). Reduced levels of NO were not due to a decrease in macrophage viability or cell numbers following treatment with the potassium channel blockers (Table 4.5).

4.2.4.3 Time-course production of NO by stimulated goldfish macrophages treated with potassium channel blockers

Goldfish macrophages were stimulated with LPS (10 μg/ml) and incubated for 72 h at 20°C in medium or in medium supplemented with 2.5 mM, 200 μM, and 10 mM of 4-AP, quinine, and TEA, respectively. After 12, 24, 48, and 72 h, supernatants were removed from the cultures and examined for NO production using the Griess reaction. As shown in Fig. 4.15, activated goldfish macrophages produced detectable levels of NO between 24 h and 48 h. In the presence of the potassium channel blockers 4-AP and TEA, NO production by macrophages was significantly reduced but still detectable after 48 h ($P<0.05$) and was also significantly reduced at 72 h ($P<0.0001$). Macrophages treated with quinine only produced detectable levels of NO after 72 h, which was significantly lower than control values ($P<0.0001$).

4.2.4.4 Relative changes of goldfish macrophage membrane potential (V_m) following treatments with potassium channel blockers

It has been previously shown that the V_m of mammalian macrophages is maintained ~~and~~ modulated predominantly by the activity of potassium channels in the plasma membrane [572]. As a result, changes in plasma membrane V_m would be representative of alterations in the function of potassium channels. To measure the effects of potassium channel blockers on goldfish macrophage plasma membrane V_m, we used the fluorescent dye bis-oxonol. Bis-oxonol slowly distributes across biological membranes according to the V_m and binds to hydrophobic components. Since the quantum yield of the dye increased significantly upon binding, the relative fluorescence (RFU) of cells incubated in medium containing the dye increased upon depolarization and decreased with hyperpolarization [517].

In the present study, it was observed that the RFU of untreated goldfish macrophages, loaded with 5 μM bis-oxonol, ranged between 60 ± 20 RFU per 5×10^4 cells when using the fluorimeter and demonstrated a wider range of fluorescence intensities when using the flow cytometer (400 ± 150 per 10,000 cells). To compensate for variations in the loading efficiency of bis-oxonol and to allow for comparisons between independent experiments, I normalized the RFU values such that the untreated control group had an RFU value of 1.0. Changes in the RFU of macrophages in medium containing bis-oxonol was an indication of relative changes in plasma membrane V_m, with a value >1.0 representing depolarization and a value < 1.0 representing hyperpolarization. Treatment with 4AP (2.5 mM), quinine (200 μM) or 10 mM TEA each significantly depolarized (RFU > 1.0; $P<0.0001$) the plasma membrane V_m compared to untreated controls (Fig. 4.16). Plasma membrane depolarization induced by the potassium channel blockers was observed at the earliest time point tested in this study (1 h) and was sustained for at least 24 h (Fig. 4.16 and Table 4.6). Compared to 4-AP and quinine, TEA caused smaller changes in goldfish macrophage V_m at all time points examined. Activation of goldfish macrophages with MAF and/or LPS did not cause any significant changes in V_m compared to control cells (data not shown).

4.2.4.5 Reversibility of plasma membrane depolarization following removal of potassium channel blockers

Treatment of goldfish macrophages with potassium channel blockers caused significant depolarization of the plasma membrane (Fig. 4.16). To determine whether this effect was directly due to treatments with the potassium channel blockers, a series of wash-out experiments were performed. Potassium channel blockers, 4-AP (2.5 mM) and quinine (200 μM), were used since they caused the most significant changes in goldfish macrophage V_m (Fig. 4.16). Shown in Fig 4.17 is the effect of a 3 h treatment with 4-AP (2.5 mM) or quinine (200 μM) followed by removal of the drug. Control cells stained with bis-oxonol demonstrated a shift in fluorescence intensity (FL-2) compared to unstained cells (Fig. 4.17A). Incubations in the presence of 4-AP or quinine caused a significant ($P<0.0001$) increase in FL-2 fluorescence (i.e. depolarization) which was reversed by washing-out the blockers. The fluorescence intensities (FL-2) of control cells, potassium channel blocker-treated, and wash-out groups are summarized (Fig. 4.17B) which demonstrated the reversibility of V_m changes caused by potassium channel blockers (Fig.17) and suggests that the potassium channel blockers are causing depolarization of the macrophage plasma membrane through an unknown mechanism and whether this is a direct or indirect effect remains to be determined.

4.2.4.6 Blocking potassium channels caused reduced respiratory burst activity of goldfish macrophages

Goldfish macrophages treated with 2.5 mM 4-AP and primed with MAF and LPS demonstrated significant reductions in ROI production after 6 and 24 h (Table 4.6). The reductions in respiratory burst activity after 6 and 24 h were 21.9 % and 39.6% (Exp 1), 17.3 % and 39.9 % (Exp 2), and 20.6% and 26.2 % (Exp 3), respectively (Table 4.6). Relative changes in macrophage V_m were simultaneously examined using bis-oxonol fluorescence. After 6 and 24 h the plasma membrane of macrophages treated with 4-AP were significantly depolarized (i.e. RFU > 1.0; $P<0.0001$) when compared to untreated controls. Potassium channel blocker-treated macrophages demonstrated a 2- to 3-fold increase in bis-oxonol RFU (i.e. depolarization) at both time points (6 and 24 h) for all three experiments (Table 4.6).

4.2.4.7 Alteration of goldfish macrophage iNOS mRNA expression by treatment with potassium channel blockers

The relative changes in iNOS mRNA expression by activated goldfish macrophages treated with potassium channel blockers using Northern blot analysis is shown in Fig.4.18A and Fig.4.18B. Figure 4.18A shows the time-course induction of iNOS after stimulation with LPS (10 μg/ml) in the absence (control; 0 mM 4-AP) or presence of 2.5 mM 4-AP. Detectable levels of iNOS expression were observed after 6 h in the control group, which peaked after 12 h, and declined slightly by 24 h. Cells treated with 2.5 mM 4-AP, which were previously shown to have significantly reduced NO production, demonstrated a relative increased expression of iNOS mRNA, which was significantly higher after 24 h. These increased levels of iNOS expression were not an artifact of RNA loading as demonstrated by the β-actin expression control. The other potassium channel blockers used in this study (e.g. quinine and TEA), also appeared not to inhibit iNOS expression after 12 and 24 h (Fig. 4.18B)

4.3 Discussion

The results presented in this chapter demonstrated that crude leukocyte supernatants derived from mitogen-stimulated goldfish kidney leukocytes contained a variety of factors that can modulate goldfish macrophage antimicrobial functions. In particular, a factor with potent macrophage deactivating properties was identified. In addition to this endogenous leukocyte-derived MDF, exogenous factors such as pharmological blockers of potassium channels, were also shown to significantly impair the production of ROI and RNI by goldfish macrophages.

Functional characterization of the leukocyte-derived MDF required that the crude leukocyte supernatants were first separated using GP-FPLC in order to segregate MAF activity from MDF activity. For this reason, supernatants collected from mitogen-stimulated leukocytes were fractionated using a Superose 6 column and individual fractions tested for their ability to inhibit NO production. Goldfish macrophages were then pre-treated with individual GP-FPLC fractions prior to stimulation with MAF and LPS, ensuring that any MDF in the individual fractions would be presented to macrophages prior to the activation signals.

Inhibition of the macrophage-mediated NO response by the leukocyte-derived MDF was time-, dose-, and temperature-dependent. Macrophages treated with MDF 6 h or 24 h prior to stimulation with crude MAF and LPS, exhibited reduced NO responses of 70% and 89%, respectively. The simultaneous addition of MDF and the activating signals caused only a 34% reduction in the macrophage NO response. Macrophages treated with the MDF 24 h after stimulation with MAF and LPS had no significant inhibition of NO production. These observations suggested that optimal MDF activity was achieved by pre-treating goldfish macrophages with the leukocyte-derived MDF prior to activation and these results are similar to those reported in murine macrophages treated with IL-4. Interleukin-4 inhibited NO production by IFNγ- and/or LPS activated macrophages provided that macrophages are pre-treated with IL-4 prior to activation with IFNγ and/or LPS [566]. However, if IL-4 was added

18 h after IFNγ and/or LPS, an enhancement of NO production and subsequent killing activities by activated murine macrophages was observed [566].

Based on biochemical characterization, the goldfish kidney leukocyte-derived MDF had an approximate MW of 5-15 kD and a pI < 4.0. Subsequent SDS-PAGE analysis of the C-FPLC purified MDF indicated that the functional protein had an approximate MW of 12 kD. Cytokines such as TGFβ (TGFβ1, -β2, and –β3) [598], IL-4 [566], and IL-10 [567] are known to deactivate mammalian macrophages. TGFβ inhibits IFNγ-induced NO production by at least three different mechanisms: 1) decreased stability of iNOS mRNA; 2) decreased translation of iNOS mRNA; and 3) increased degradation of iNOS protein after iNOS has been expressed [598]. Interleukin-10 inhibited both the release of TNFα by activated macrophages and MAFs derived from T cells [567, 599]. Bovine TGFβ1 has been shown to inhibit ROI production of rainbow trout macrophages suggesting that this cytokine may be an important macrophage deactivator in fish [327]. Recently, TGFβ from carp, rainbow trout, bass, and plaice have been cloned, using degenerate oligonucleotide primers and RT-PCR [591-592, 600-602]. In zebrafish, both TGFβ1 and its type II receptor have also been cloned [603]. Additionally, a partial sequence for goldfish TGF-β1/5 has also been reported [593]. Carp TGFβ2 shares 81% and 93% nucleotide sequence homolgy with human TGFβ2 in the precursor and the mature regions of the molecule, respectively. The high conservation of piscine and mammalian TGFβ, the potent macrophage deactivating properties of this cytokine, and the similar MW of the biologically active forms of mammalian TGFβ1-3 and the goldfish leukocyte-derived MDF characterized in this chapter, suggested that TGFβ may be a potential candidate for this endogenously-derived teleost MDF. However, a mammalian anti-TGFβ polyclonal antibody did not demonstrate immunoreactivity by Western blot analysis when tested against the purified teleost MDF (data not shown). This indicated that either the antibody does not recognize teleost TGFβ, or that other teleost molecules (i.e. equivalent to the deactivating mammalian cytokines IL-4, IL-10, and IL-13) are responsible for the macrophage deactivation activities observed in this study. The precise molecular nature of this goldfish MDF remains to be determined and future studies should focus on the cloning of the goldfish TGFβ gene and biochemical chracterization of the recombinant protein. In addition antibodies generated against recombinant goldfish TGFβ should be tested against the purified 'native' MDF.

My results also demonstrated that the deactivation of goldfish macrophages can occur in response to exogenous factors and for the first time I showed that fish macrophage antimicrobial functions are affected by the pharmological blocking of potassium channel activity. Compared to the information regarding mammalian macrophage potassium channels, relatively little is know about the types of potassium channels found on fish macrophages. Therefore, pharmacological blockers that are known to affect numerous types of potassium channels, through a variety of different mechanisms were chosen for these experiments. For example, the pharmological blocker TEA acts by binding to a specific receptor site on the extracellular side of the potassium channel and physically blocks the pore [571, 604-605]. Delayed-rectifier type, inward rectifier type, calcium-sensitive type and ATP-sensitive type potassium channels are all affected by TEA. The inhibitor 4-AP is lipid-soluble and membrane permeable, which crosses plasma membranes to reach its site of action [571]. Within macrophages, 4-AP physically blocks the pore of the delayed-rectifier type and voltage-sensitive potassium channels. Quinine is an alkaloid isolated from plants of the *Cinchona* family and like 4-AP, is a membrane-permeant molecule that blocks potassium channels by passing into the cytoplasm of cells or diffusing from the cell membrane to bind the receptor within the pore and physically block the passage of potassium ions [606-608]. Quinine blocks delayed-rectifier, calcium-sensitive, and ATP-sensitive type potassium channels of macrophages. All of these blockers significantly inhibited goldfish macrophage activation. The wide variety of potassium channels that are likely present on goldfish macrophages suggests that each type contributes a small amount to activation processes. Interestingly, TEA failed to significantly inhibit NO production by goldfish macrophages stimulated with MAF alone or in combination with LPS, but significantly inhibited NO production by macrophages stimulated with LPS alone. Conversely, quinine and 4-AP inhibited NO production by cells stimulated with LPS and/or MAF. This suggests that the activation pathway(s) triggered by LPS and MAF may be different and appeared to be mediated by different types of potassium channels.

The major role of potassium channels within cells is the establishment and maintenance of plasma membrane potential (V_m) [572, 609]. Alterations in the activity of potassium channels results in changes in V_m (i.e. depolarization or hyperpolarization), which could have significant effects on proteins and enzymes embedded in the plasma membrane [610]. Modulation of the activities of functional enzymes found in the plasma membrane can subsequently alter immune cell functions. For instance, potassium channel activity was shown to mediate the activity of the membrane bound NADPH oxidase of human macrophages [574]. Activation of this enzyme, which catalyzes superoxide formation and production of ROI, was dependent on V_m that was directly modulated by the opening of calcium-dependent potassium channels. In addition, TEA inhibited the respiratory burst response in microglia and charybdtoxin dramatically inhibited the respiratory burst by cultured macrophages stimulated by opsonized zymosan [574, 611]. These findings further supported the hypothesis that potassium channels regulate NADPH oxidase activity but little is known about the mechanism of enzyme inhibition.

My results also demonstrated that potassium channel blockers inhibited the production of NO by fish macrophages, an effect that we have also demonstrated in murine macrophages [569-570]. I hypothesized that reduced NO production by potassium channel blocker-treated goldfish macrophages was due to the reduced expression of iNOS mRNA. Following treatments with potassium channel blockers, the expression of goldfish macrophage iNOS mRNA levels were surprisingly increased. These results suggested that the significant reductions of NO production induced by blockage of potassium channels, were not due to suppressed iNOS mRNA levels. The observed increased levels of iNOS mRNA levels and subsequent reduction in NO production induced by potassium channel blockers may be explained by postulating the absence of a negative regulatory feedback mechanism. Regulation of iNOS enzyme synthesis and RNA transcription are tightly controlled processes [411]. Recently, it has been demonstrated that the end-products produced by iNOS enzyme activity (i.e. NO and peroxynitrite) can inhibit iNOS mRNA expression, iNOS enzyme synthesis, and iNOS enzyme activity [612-614]. Nitric oxide generated intracellularly following iNOS induction or released from NO donors, limited the transcription of the iNOS gene. Interestingly, removal of the negative feedback signal (i.e. NO) by inhibition of iNOS enzyme activity, resulted in an increase of iNOS mRNA expression [612]. Thus, the observed increase of goldfish iNOS mRNA expression after treatment with potassium channel blockers may be due to the impairment of the negative feedback signal, due to significantly reduced levels of NO that were produced.

In addition to reducing ROI and RNI production by activated goldfish macrophages, I also demonstrated that treatment of goldfish macrophages with blockers of potassium channel activity caused subsequent depolarization of the plasma membrane, which may lead to impairment of intracellular signaling steps required for goldfish macrophage activation. Recently, a direct link has been established between LPS-activation of macrophages, potassium channel activity and transmembrane signaling [615]. This report demonstrated that activation of potassium channels was an early step in the transmembrane signal transduction in macrophages; which could be inhibited by potassium channel blockers. Therefore, modulation of potassium channels alters LPS-induced transmembrane signaling required for gene transcription (i.e. TNFα). This recent report may explain why treatment of human alveolar macrophages with quinine prevented LPS-induced TNFα release and the findings of another group that showed that the release of TNFα by LPS-stimulated mouse macrophages was dependent on V_m [575, 616]. A similar cascade of events may also occur in fish macrophages. Goldfish macrophages respond to LPS stimulation by up-regulating the expression of a variety of genes (i.e. iNOS, TNFα, and IL-1). Reduced production of cytokines and antimicrobial factors (i.e. NO and ROI) by fish macrophages, following perturbation of potassium channels would significantly impair their antimicrobial functions. Further investigations are required to fully understand the role of potassium channels in teleost immune cell function and potentially identify other factors (endogenous and/or exogenous) that may alter potassium channel activity.

Table 4.1. Effect of mitogen stimulation and/or mixed lymphocyte reactions on the production of MAF supernatants by goldfish kidney leukocytes[1].

Fish	Treatment	Nitrite (µM) ± SEM
1	none	-0.26 ± 0.04
	mitogen	2.25 ± 0.07
2	none	-0.07 ± 0.04
	mitogen	3.75 ± 0.10
3	none	0.04 ± 0.04
	mitogen	5.56 ± 0.11
4	none	0.55 ± 0.11
	mitogen	2.25 ± 0.07
5	none	0.40 ± 0.04
	mitogen	0.22 ± 0.06
MLR[2]	none	4.51 ± 0.04
	mitogen	3.35 ± 0.13

[1] Goldfish kidney leukocytes were isolated from individual fish and cultured in 24 well tissue culture plates at a concentration of 4×10^6 cells/well. Cells were cultured in the absence or presence of mitogens (10 µg/ml Con A, 10 ng/ml PMA, and 100 ng/ml calcium ionophore A23187) in serum-free medium for 72 h at 20°C before supernatants were collected and tested for MAF activity. MAF activity was determined by co-stimulating IVDKM (5×10^4 cells/well) with a 1:4 dilution of crude MAF and LPS (1 µg/ml). Nitrite content within individual wells was determined using the Greiss reaction 72 h after stimulation. Data represents the mean ± SEM of triplicate cultures. IVDKM stimulated with LPS alone produced –0.31 ± 0.20 µM nitrite.

[2] Mixed lymphocyte reactions (MLR) were generated by pooling kidney leukocytes from 20 fish and culturing the cells in 75 cm^2 tissue culture flasks in serum-free medium at a concentration of 4×10^6 cells/ml. Cells were cultured in the absence or presence of mitogens (at the concentrations listed above) for 72 h at 20°C before supernatants were collected and tested for MAF activity as described above.

Table 4.2. NO-inducing activity of GP-FPLC fractionated MAF supernatants[1].

Sample	Nitrite (μM) ± SEM
MAF 1[2]	7.78 ± 013
MAF 2[3]	5.89 ± 0.13
Unstimulated[4]	1.31 ± 0.39

[1] Samples were concentrated approximately 30-fold using microcentrifugal concentrators and fractionated using a Superose 6 GP-FPLC column. Individual fractions were then tested for MAF activity by seeding $5x10^4$ IVDKM into wells of a 96 well tissue culture plate and stimulating the cells with a 1:4 dilution of GP-FPLC fraction #34 and 1 μg/ml LPS. Nitrite production was determined 72 h later using the Griess reaction. Listed in the table are the results (mean ± SEM of triplicate cultures) obtained from the fraction with maximal NO-inducing activity (i.e. fraction #34). IVDKM stimulated with LPS alone produced 1.81 ± 0.32 μM nitrite.

[2] MAF 1 was obtained by pooling the mitogen-stimulated supernatants from Fish 1-3 in Table 4.1. Note that the supernatants from these individual fish displayed strong nitric oxide-inducing activity (Table 4.1), which was retained following GP-FPLC fractionation.

[3] MAF 2 was obtained by pooling the mitogen-stimulated supernatants from Fish 4 and 5 in Table 4.1. Note that the supernatants from these individual fish did not induce NO-inducing activity (Table 4.1), but following GP-FPLC fractionation, displayed activity.

[4] Unstimulated samples were obtained by pooling the unstimulated cultures from Fish 1-6 (Table 4.1). Note that no NO-inducing activity was observed in either the individual cultures (Table 4.1) or the pooled and GP-FPLC fractionated sample.

Table 4.3. Effect of leukocyte-derived MDF on viability, total cell number, and NO production by goldfish macrophages.[1]

Treatment	Cell Number (x10^4)	% Viable[2]	Nitrite (µM)
Medium	50 ± 2.1	88	1.1 ± 0.1
PBS	55 ± 2.8	91	13.4 ± 0.1
MDF	55 ± 1.2	90	6.1 ± 0.1

[1] Viability, cell number and nitric oxide production of IVDKM were determined according to the procedure described in materials and Methods (Chapter 2).
[2] % Viable Cells =[(Number of live cells – Number of Dead cells) / Number Live cells]x100.

Table 4.4. Time-dependency of MDF-mediated inhibition of NO[1]

Time of exposure to MDF	n	Control[2] (± SEM)	MDF-Treated Nitrite (± SEM)	% Inhibition[3]	P-value
Pre-treatment					
24 hours	7	10.7 ± 2.9	1.2 ± 0.4	89	0.0940
6 hours	17	12.4 ± 1.1	3.7 ± 0.5	70	0.0001
0 hours	10	11.2 ± 2.2	7.4 ± 1.3	34	0.0347
Post-treatment					
6 hours	5	7.1 ± 2.0	8.2 ± 1.8	-15[4]	0.0567

[1] Goldfish IVDKM (5 x 10^4 cell/well) were pre-treated with MDF (1:3 dilution) at the various times indicated, pre- and post-stimulation, with crude MAF (1:4 dilution) and LPS (1 µg/ml). Cells incubated 72 h at 20°C prior to determination of NO production.

[2] Control represents goldfish macrophages activated with MAF and LPS and treated with 1xPBS (1:3) dilution at the various times indicated instead of MDF.

[3] % Inhibition =[(Control Nitrite-Treated Nitrite) / Control Nitrite] x 100.

[4] Negative values represent an increase in nitrite production when compared to control groups, which were not exposed to MDF.

Table 4.5. Effect of potassium channel blockers on viability and total cell number of goldfish macrophages

Treatment[1]	Incubation Time (h)	Cell Number $(x10^4)$	% Viable[2]	P value[3]
Control	24	31 ± 1	> 95	-
	48	26 ± 2	> 95	-
	72	34 ± 1	> 95	-
2.5 mM 4-AP	24	31 ± 2	> 95	1.000
	48	31 ± 1	> 95	0.114
	72	31 ± 3	> 95	0.365
10 mM TEA	24	34 ± 1	> 95	0.342
	48	30 ± 3	> 95	0.174
	72	31 ± 1	> 95	0.378
200 μM Quinine	24	34 ± 2	> 95	0.319
	48	31 ± 2	> 95	0.109
	72	30 ± 1	> 95	0.141

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[1] Highest doses of potassium channel blockers (i.e. 2.5 mM 4-AP, 10 mM TEA, and 200 μM Quinine) with maximal inhibitory activity were chosen for viability experiment.

[2] Trypan blue exclusion was used for determination of cell numbers and macrophage viability using the formula: %Viable cells=[number of live cells-number of dead cells)/number of live cells]x100

[3] P value when comparing potassium channel blocker treated cells with the control group of cells.

Table 4.6. Effect of 4-AP on the respiratory burst response and V_m of goldfish macrophages

		Respiratory burst[1]			V_m[2]	
	Time (h)	0 mM 4-AP	2.5 mM 4-AP	P value[3]	Bis-oxonol (RFU)[4]	P value[5]
Exp 1	6	0.14 ± 0.01	0.11 ± 0.01	0.0119	2.12 ± 0.01	0.0001
	24	0.16 ± 0.01	0.10 ± 0.00	0.0001	2.34 ± 0.07	0.0001
Exp 2	6	0.34 ± 0.02	0.28 ± 0.02	0.0125	1.94 ± 0.01	0.0001
	24	0.27 ± 0.01	0.16 ± 0.00	0.0001	2.02 ± 0.05	0.0001
Exp 3	6	0.26 ± 0.01	0.21 ± 0.01	0.0001	2.62 ± 0.05	0.0001
	24	0.19 ± 0.01	0.14 ± 0.01	0.0001	3.11 ± 0.01	0.0001

[1] Respiratory burst response of goldfish macrophages was determined using the NBT assay as described in the materials and methods.

[2] Relative changes of goldfish macrophage membrane potential (V_m) was determined using the dye bis-oxonol as described in the materials and methods.

[3] P-value represents the significant reduction of respiratory burst response of goldfish macrophages following treatment with 2.5 mM 4-AP.

[4] Relative fluorescence units (RFU) represent changes in relative fluorescence intensity (FL-2) of bis-oxonol-loaded goldfish macrophages following treatment with the potassium channel blocker 4-AP. Data normalized to the RFU of control cells (0 mM 4-AP) by dividing RFU of treated cells by RFU of control cells. Following normalization of RFU values, control macrophage RFU's were 1.0. See results section for significance of changes in bis-oxonol fluorescence intensities.

[5] P-value represented the significant increase in bis-oxonol RFU by macrophages treated with 4-AP.

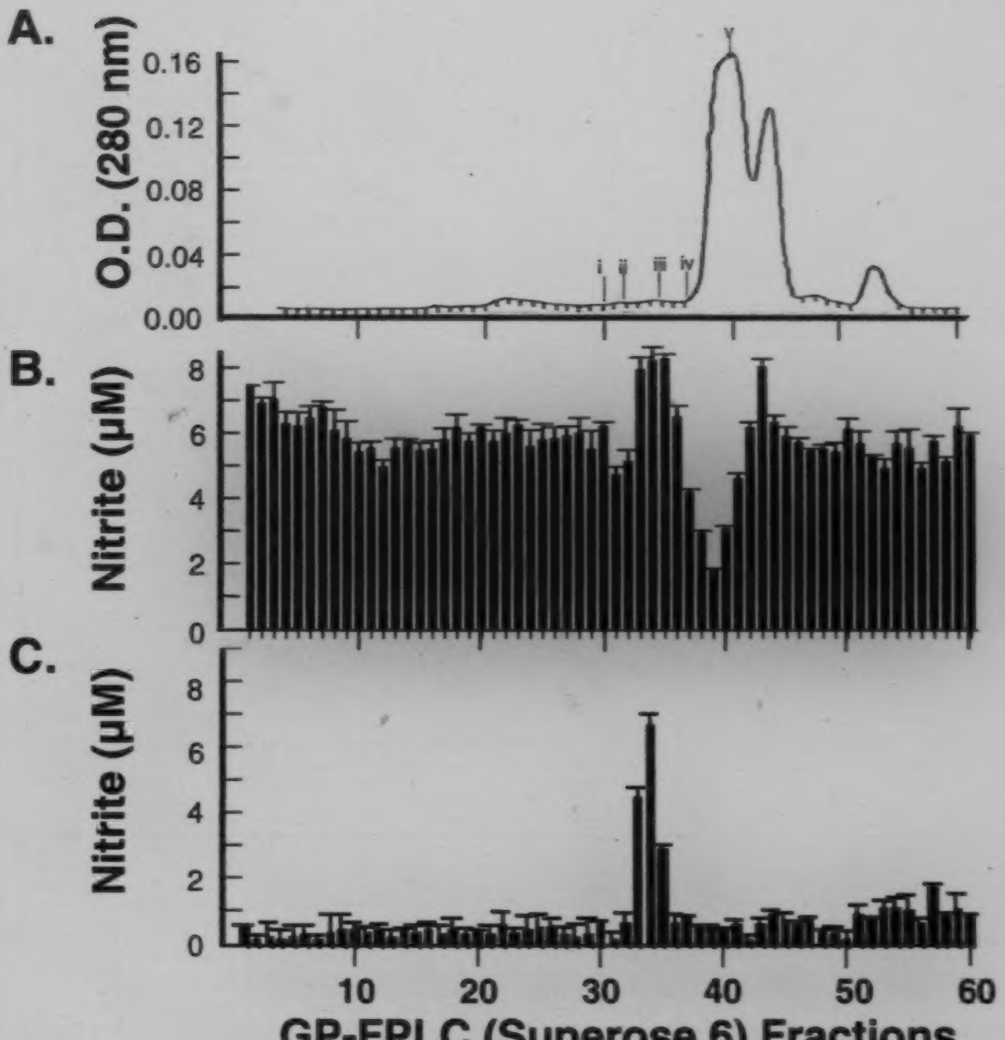

Figure 4.1. Identification of macrophage activating and deactivating activities present in crude leukocyte supernatants. Supernatants were separated according to size using a Superose 6 column (A), and activity was determined in each fraction using the Griess reaction. For the detection of MDF activity (B), IVDKM were seeded into the wells of half-area 96-well culture plates (5x10^4 cells/well) and pre-treated for 6 h with individual Superose 6 fractions (1:3 v/v) for 6 h prior to activation with crude MAF (1:4 v/v) and LPS (1 µg/ml). MAF activity was detected by treating IVDKM (5x10^4 cells/well) with individual Superose 6 fractions (1:4 v/v) in conjunction with LPS (1 µg/ml) (C). Nitrite production was measured after 72 h incubation at 20°C. Each bar represents the mean ± SEM of triplicate cultures, and is representative of three independent experiments that were performed. Note: (i) Alcohol Dehydrogenase 150 kD; (ii) Bovine Serum Albumin 66 kD; (iii) Carbonic Anhydrase 29 kD; (iv) Cytochrome C 12.4 kD; (v) Aprotinin 6 kD.

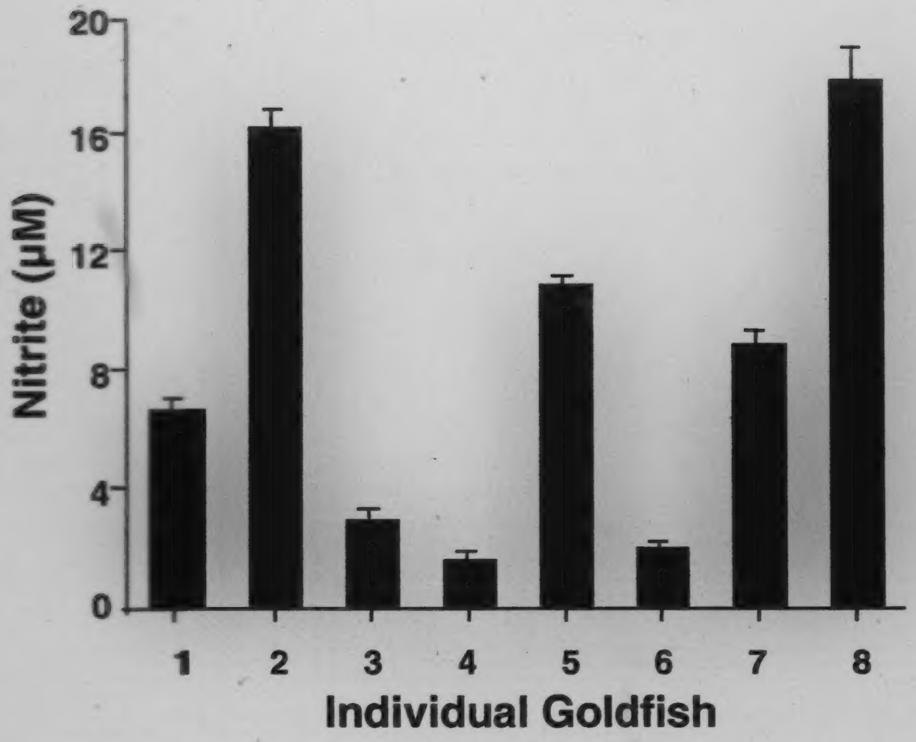

Figure 4.2. Fish to fish variability in response to NO-inducing activity of crude leukocyte supernatants. Eight individual goldfish were used for the preparation of IVDKM cultures, which were subsequently seeded into the wells ($5x10^4$ cells/well) of a 96-well half-area culture plate. Each IVDKM culture was stimulated with an identical sample of crude MAF (1:4 v/v) in conjunction with 1 µg/ml of LPS. Cells were incubated at 20°C for 72h prior to the determination of nitrite production. Each bar represents the mean ± SEM of triplicate cultures and the data are from a representative experiment of two independent experiments that were performed.

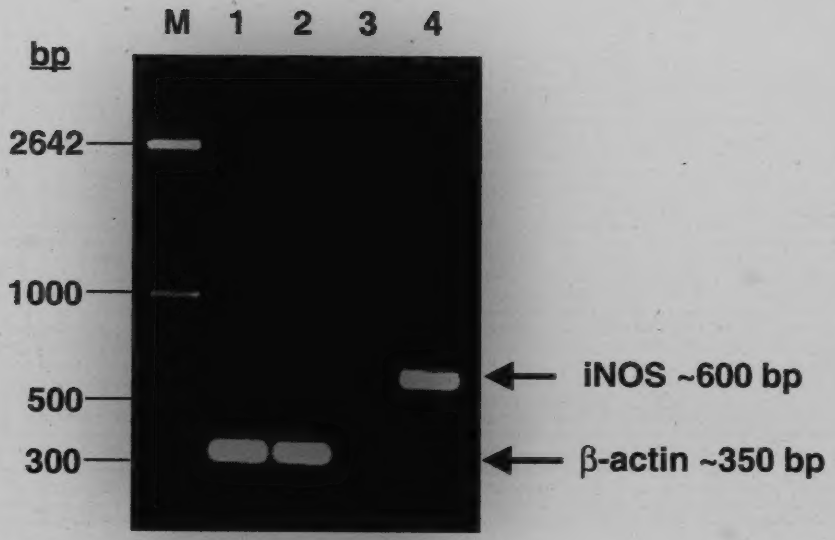

Figure 4.3. RT-PCR analysis of iNOS and β-actin mRNA expression by stimulated IVDKM. Day 8-12 IVDKM were seeded into 6 ml polypropylene tubes (5×10^6 cells/tube) and stimulated with MAF (1:4 v/v) and LPS (1 μg/ml), or were incubated in culture medium alone (unstimulated) for 6 h prior to isolation of RNA using TriZol reagent. Total RNA (2.5 μg) was used for first strand cDNA synthesis and iNOS and β-actin primers were used in the PCR reactions. PCR products (10 μl) were separated on a 1.2% agarose gel stained with EtBr. M=size marker; Lane 1=unstimulated IVDKM β-actin expression; Lane 2=stimulated IVDKM β-actin expression; Lane 3=unstimulated IVDKM iNOS expression; Lane 4=stimulated IVDKM iNOS expression.

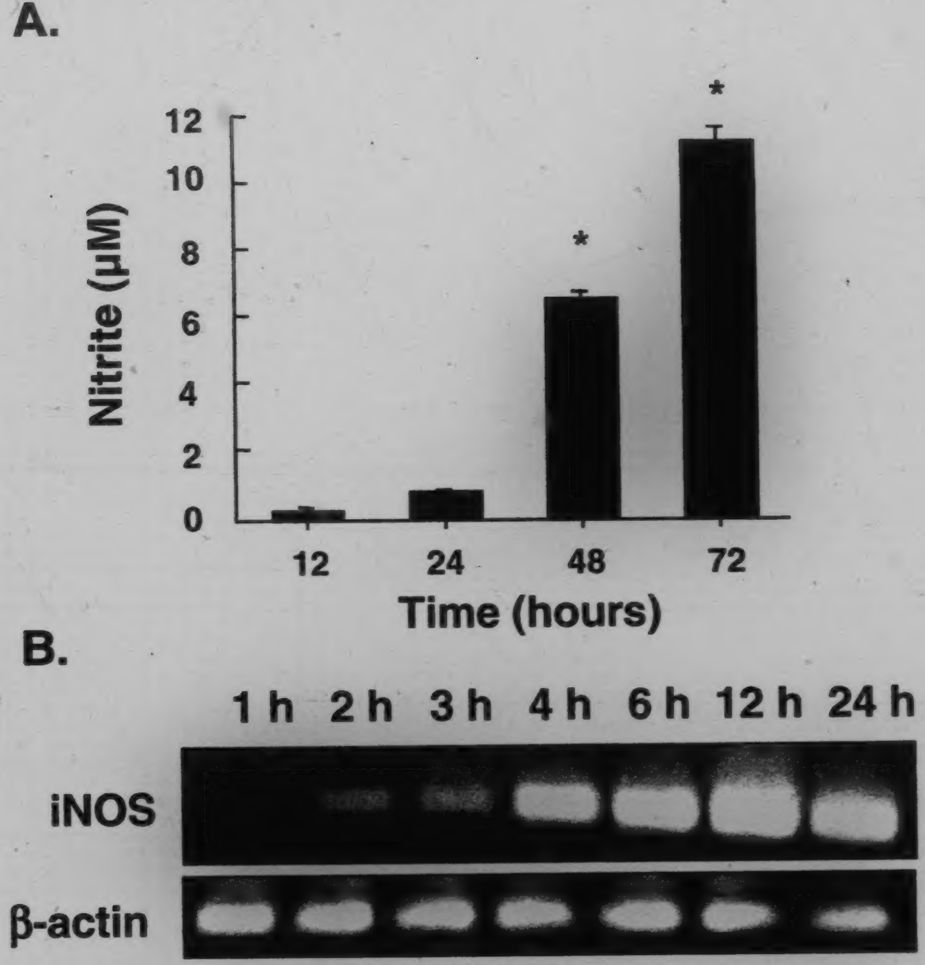

Figure 4.4. Time-course induction of NO production and iNOS mRNA expression by stimulated IVDKM. Goldfish IVDKM were seeded into the wells of half-area 96-well culture plates ($5x10^4$ cells/well) and stimulated with crude MAF (1:4 v/v) and LPS (1 mg/ml). After 12, 24, 48, and 72 h post-stimulation, supernatants were removed and measured for the presence of nitrite using the Griess reaction (A). Induction of iNOS mRNA expression was also determined using RT-PCR (B). IVDKM ($5x10^6$) were seeded into 6 ml polypropylene tubes and stimulated with MAF (1/4 v/v) and LPS (1 μg/ml). At the times indicated, RNA was extracted from the cells using TriZol reagent and PCR performed using iNOS and β-actin specific PCR primers. Bars represent the mean ± SEM of triplicate cultures and data are from a representative experiment of two independent experiments that were performed. * $P<0.05$ vs. 12 h time-point

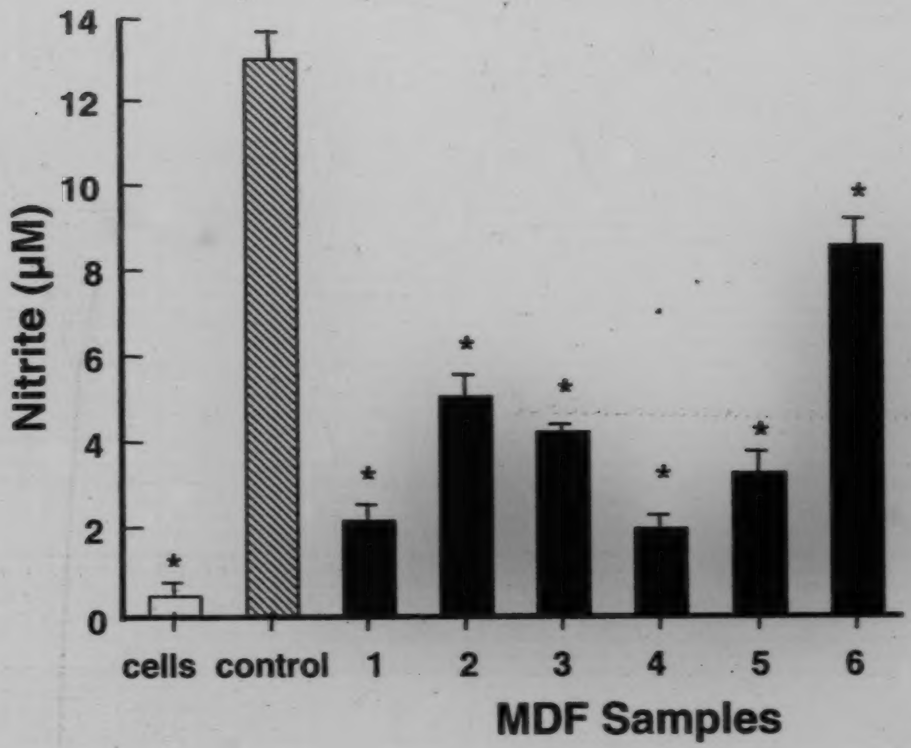

Figure 4.5. Variability of MDF activity. Six individual crude leukocyte supernatant preparations were separated on a Superose 6 column. IVDKM were seeded into the wells of half-area 96-well culture plates (5x10^4 cells/well) and pre-treated for 6 h with Superose 6 fraction #39 (1:3 v/v) for 6 h prior to activation with crude MAF (1:4 v/v) and LPS (1 µg/ml) (black bars). Control groups consist of cells pre-treated with GP-FPLC running buffer (i.e. 1 x PBS; thatched bar) 6 h prior to stimulation with crude MAF (1:4 v/v) and LPS (1 µg/ml). Cells were incubated at 20°C for 72 h prior to the determination of nitrite production. Each bar represents the mean ± SEM of triplicate cultures and the data are from a representative experiment of three independent experiments that were performed. *P<0.05 vs. control.

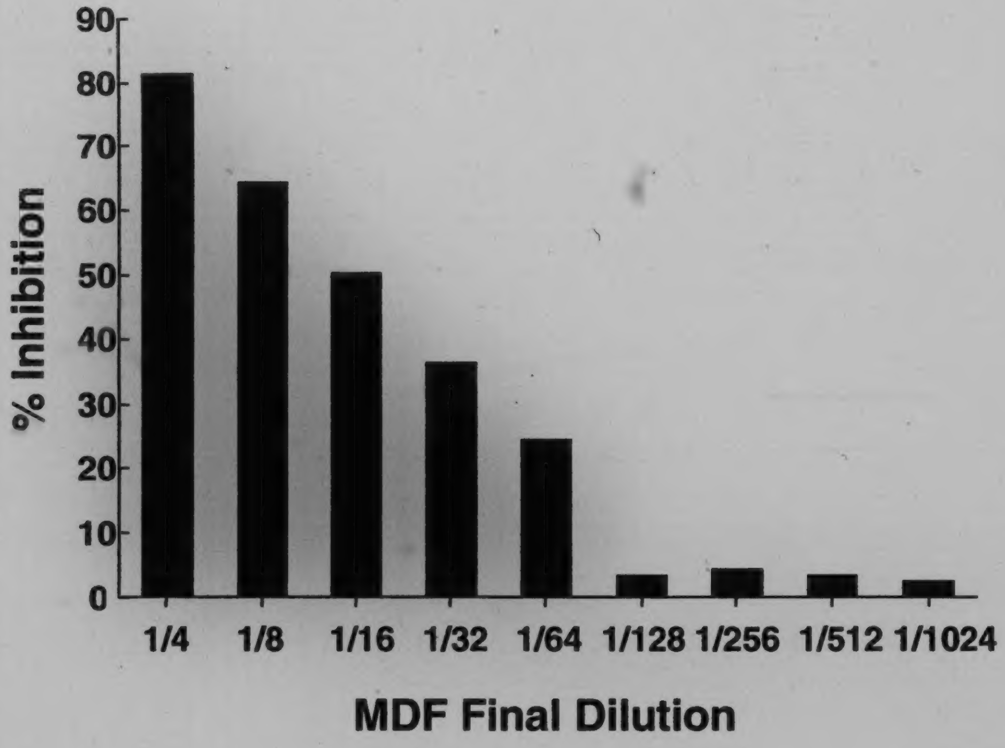

Figure 4.6. Dose-dependent inhibition of NO production by IVDKM treated with partially purified, leukocyte-derived MDF. IVDKM (5 x 10^4 cells/well) were seeded into half-area 96-well culture plates and pre-treated for 6 h with various MDF (i.e. Superose 6 fraction #39) concentrations (1:4 to 1:1024 v/v final concentration). Cells were subsequently activated with MAF (1:4) and LPS (1 μg/ml) and incubated for 72 h at 20°C prior to determination of nitrite production. Each bar represents the % inhibition nitrite production of triplicate cultures and is representative of three independent experiments that were performed.

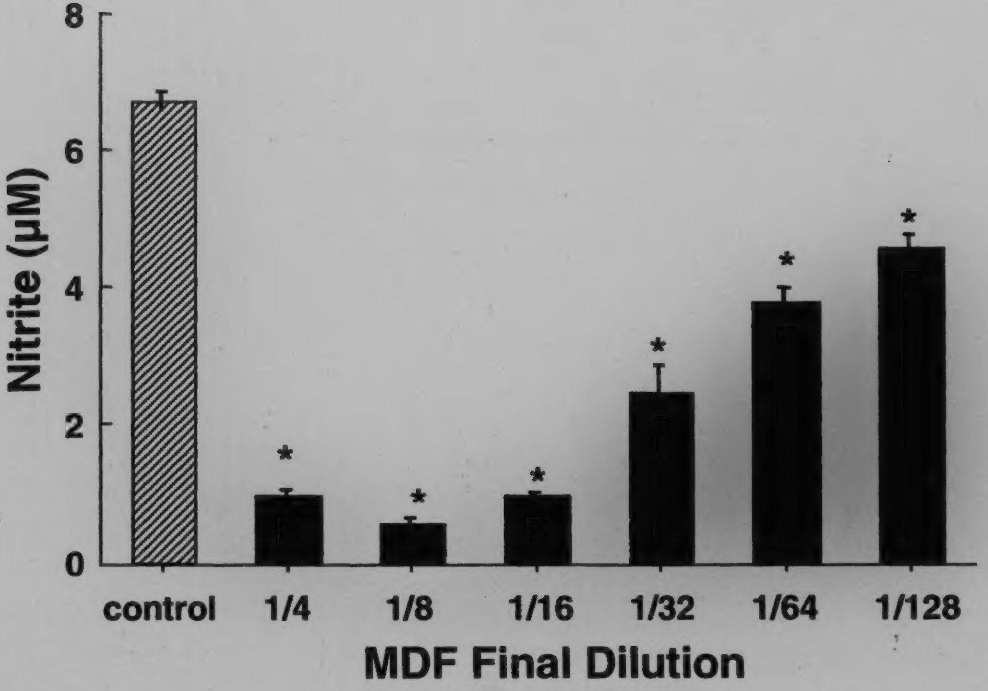

Figure 4.7. Induction and subsequent MDF-mediated inhibition of NO production by goldfish IVDKM infected with the protozoan parasite *L. major*. IVDKM (5 x 10^4 cells/well) were seeded into half-area 96-well culture plates and were then treated with various concentrations of MDF (Superose 6 fraction #39 at a 1:4 to 1:128 v/v final concentration; black bars) or column running buffer (1xPBS; thatched bar) 6 h prior to addition of parasite. *L. major* promastigotes were subsequently added to all treatment groups at 2.5 promastigotes to 1 macrophage and cells were incubated for a further 72 h at 20°C prior to determination of nitrite production. Control represents cells pre-treated with 1xPBS and then incubated with promastigotes alone. Each bar represents the mean ± SEM of triplicate cultures and is representative of two independent experiments that were performed.*$P<0.05$ vs. control.

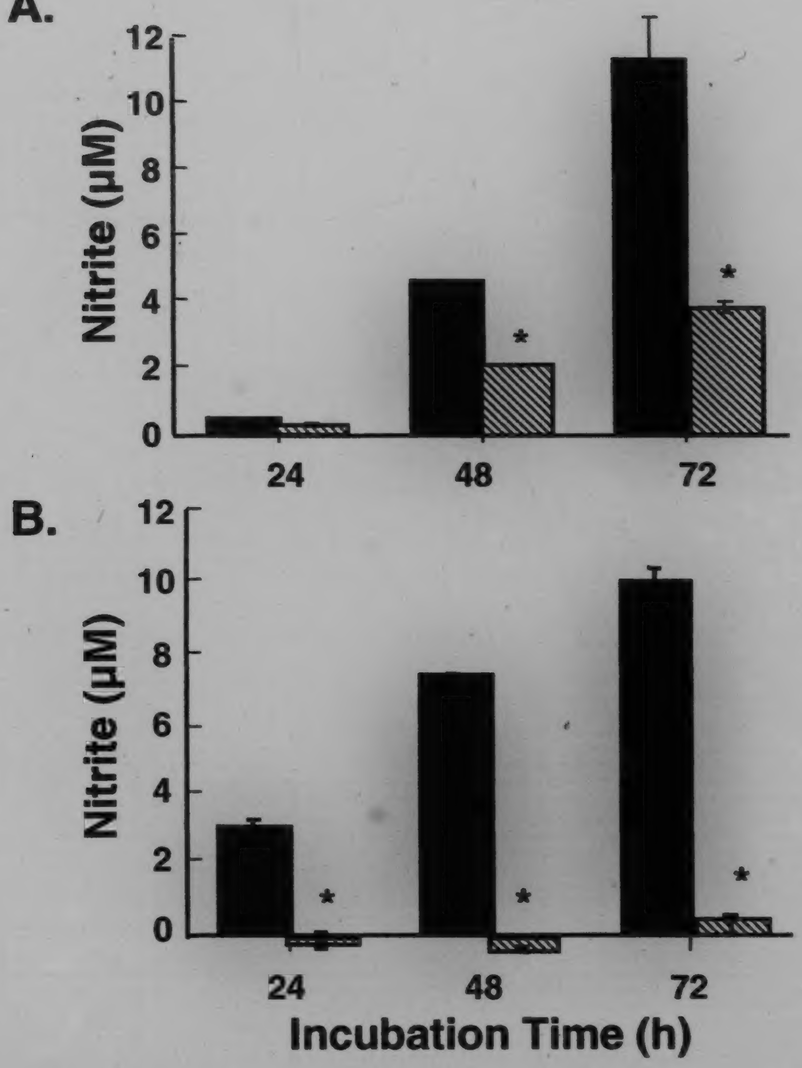

Figure 4.8. Temperature-dependency of MDF-induced inhibition of NO producion. IVDKM (5 x 10^4 cells/well) were seeded into the wells of half-area 96-well culture plates and pre-treated for 6 h with Superose 6 running buffer (1x PBS at a 1:4 v/v final dilution; black bars) or with Superose 6 fraction #39 (MDF at a 1:4 v/v final dilution; hatched bars). After 6 h pre-treatment, cells were stimulated with crude MAF (1:4 v/v) and LPS (1 µg/ml) and then incubated at 20°C (A) or 30°C (B). Nitrite production was subsequently determined at 24, 48, and 72 h after activation using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and data are from a representative experiment of two independent experiments that were performed. *$P<0.05$ vs. control group at same time point.

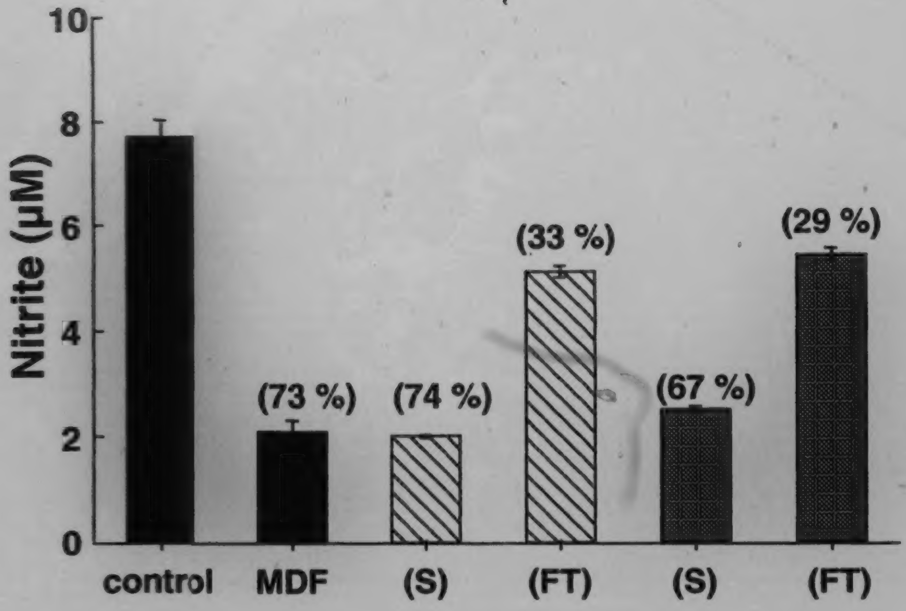

Figure 4.9. Recovery of MDF activity following microcentrifugal concentration of samples. Purification of goldfish MDF included a concentration step using microcentrifugal concentrators with a 3 kD or 10 kD MWCO membrane. To ensure that MDF activity was retained in the sample chamber (S), samples from both 3 kD (hatched bars) and 10 kD (grey bars) concentrators were tested for NO-inhibitory activity following microcentrifugal concentration. Samples from the flow through (FT) chambers that contain proteins < 3 kD (hatched bar) and < 10 kD (grey bar) were also tested. IVDKM ($5x10^4$/well) were seeded into 96-well half-area plates and pre-treated for 6 h with 1 x PBS, Superose 6 fraction #39 (MDF), or samples from the (S) chamber or (FT) chambers of the microcentrifugal concentrators (Note: concentrated samples were diluted back to the original MDF that was originally applied to the concentrators so that the final dilution for all treatments was 1:4 v/v). After 6 h cells were stimulated with MAF (1:4 v/v) and LPS (1 µg/ml) and incubated at 20°C for 72 h prior to determination of nitrite production. Control represents cells pre-treated with 1xPBS (i.e. no MDF) for 6 h then activated with MAF and LPS as described above. Each bar represents the mean ± SEM of triplicate cultures and data are from a representative experiment of three independent experiments that were performed.

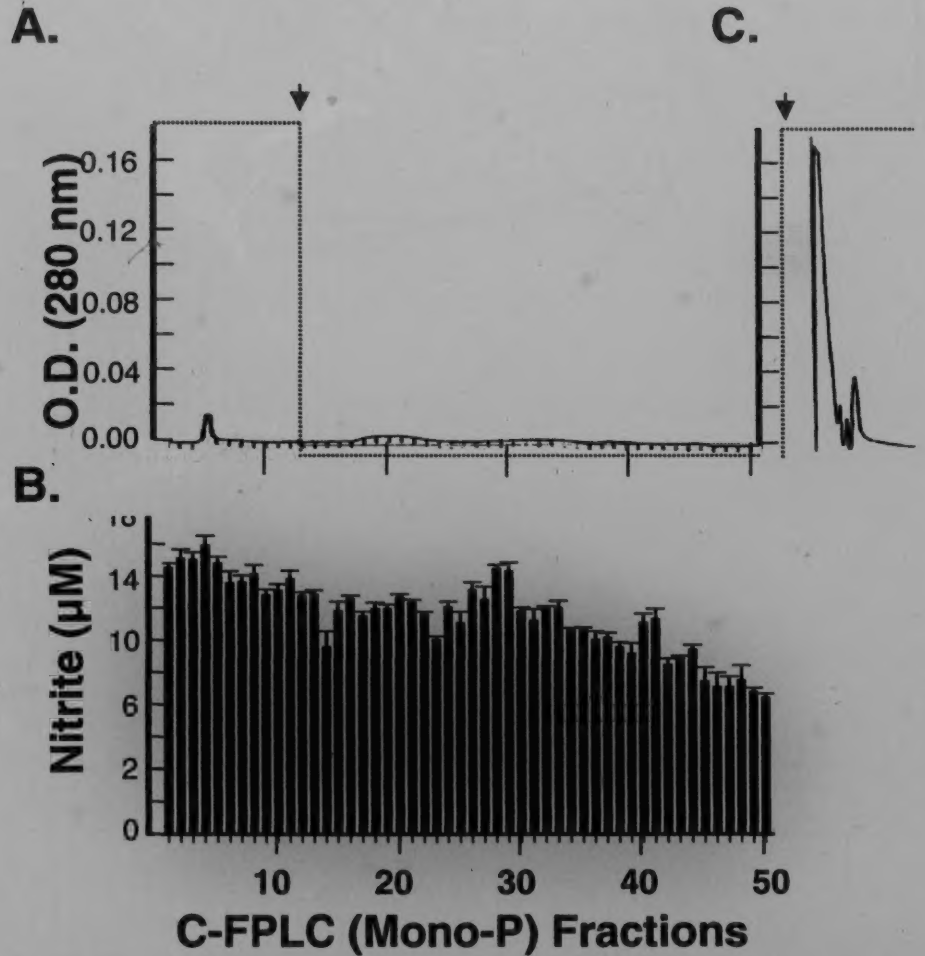

Figure 4.10. C-FPLC purification of goldfish leukocyte-derived MDF. Superose 6 fraction #39 (MDF) was separated according to its isoelectric point using a Mono-P column (A). A linear descending pH gradient (7.0-4.0) was established by running a 1:10 dilution of Polybuffer 74 at a flow rate of 0.75 ml/min (arrow indicates initiation of the pH gradient). MDF activity was determined in each fraction using the Griess reaction (B). IVDKM were seeded into the wells of half-area 96-well culture plates ($5x10^4$ cells/well) and pre-treated for 6 h with individual Mono-P fractions (1:4 v/v final dilution) for 6 h prior to activation with crude MAF (1:4 v/v) and LPS (1 µg/ml). Proteins bound to the column (pI < 4.0) were eluted with 2 M NaCl as indicated by arrow at a flow rate of 0.25 ml/min (C). Each bar represents the mean ± SEM of triplicate cultures, and is representative of three independent experiments that were performed.

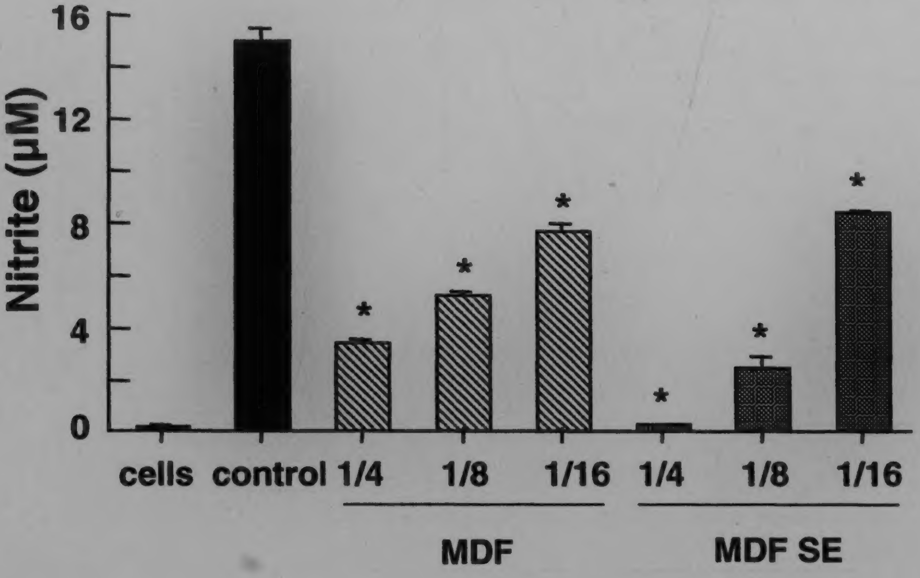

Figure 4.11. Comparison of MDF activity following GP-FPLC and C-FPLC purification. Superose 6 fraction #39 with maximal activity (MDF) was compared with the NO-inhibitory activity demonstrated by the salt-eluted C-FPLC fraction (MDF SE). Salt-eluted samples were dialyzed overnight in 1xPBS to remove excess salt prior to testing for activity. IVDKM ($5x10^4$/well) were pre-treated with medium (cells), 1 x PBS (control; 1:4 v/v final dilution), MDF (1:4-1:16 v/v final dilution), or MDF SE (1:4-1:16 v/v final dilution) for 6 h. After 6 h cells were stimulated with MAF (1:4 v/v) and LPS (1 µg/ml) and cells were incubated for a further 72 h at 20°C prior to determination of nitrite production. Each bar represents the mean ± SEM of triplicate cultures, and is representative of three independent experiments that were performed. *P<0.05 vs. control.

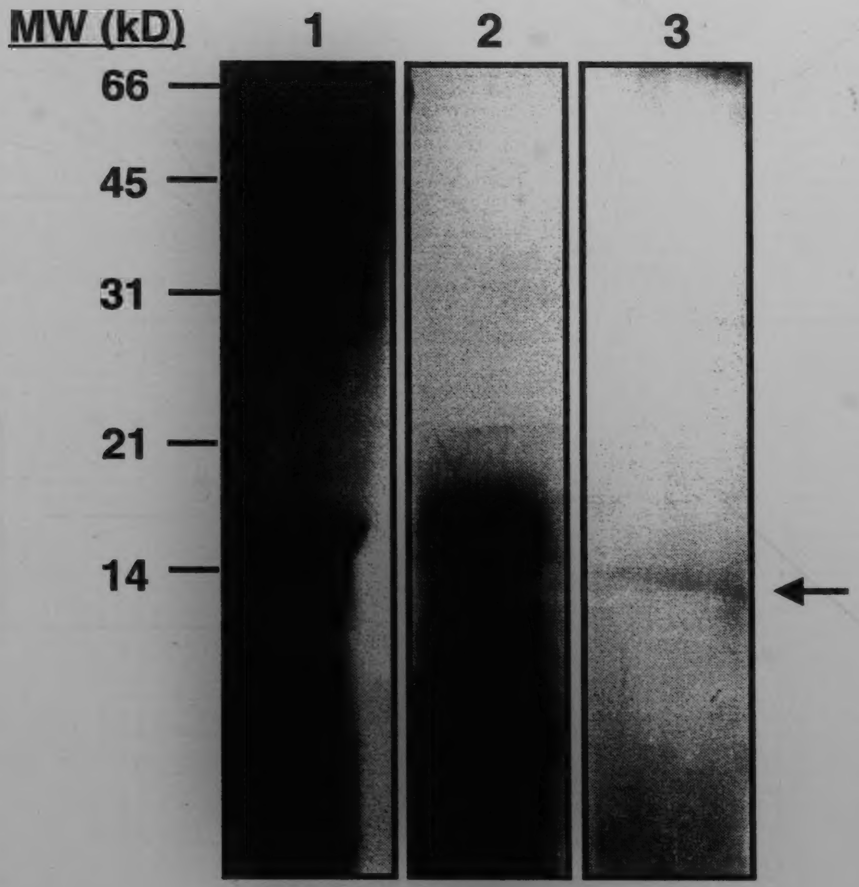

Figure 4.12. SDS-PAGE (reducing) analysis of crude leukocyte supernatants, GP-FPLC partially purified MDF, and C-FPLC purified MDF. Ten µl aliquots of crude MAF (lane 1), GP-FPLC partially purified MDF (Superose 6 fraction #39; lane 2), and C-FPLC purified MDF (Mono-P salt eluent; lane 3) were electrophoresed through 5% stacked, 20% continous SDS-PAGE gel under reducing conditions, and proteins subsequently visualized by silver staining (BioRad). Arrow indicates presence of a protein with an approximate MW of 12 kD detected in the Mono-P salt eluent that exhibited potent NO-inhibiting activity.

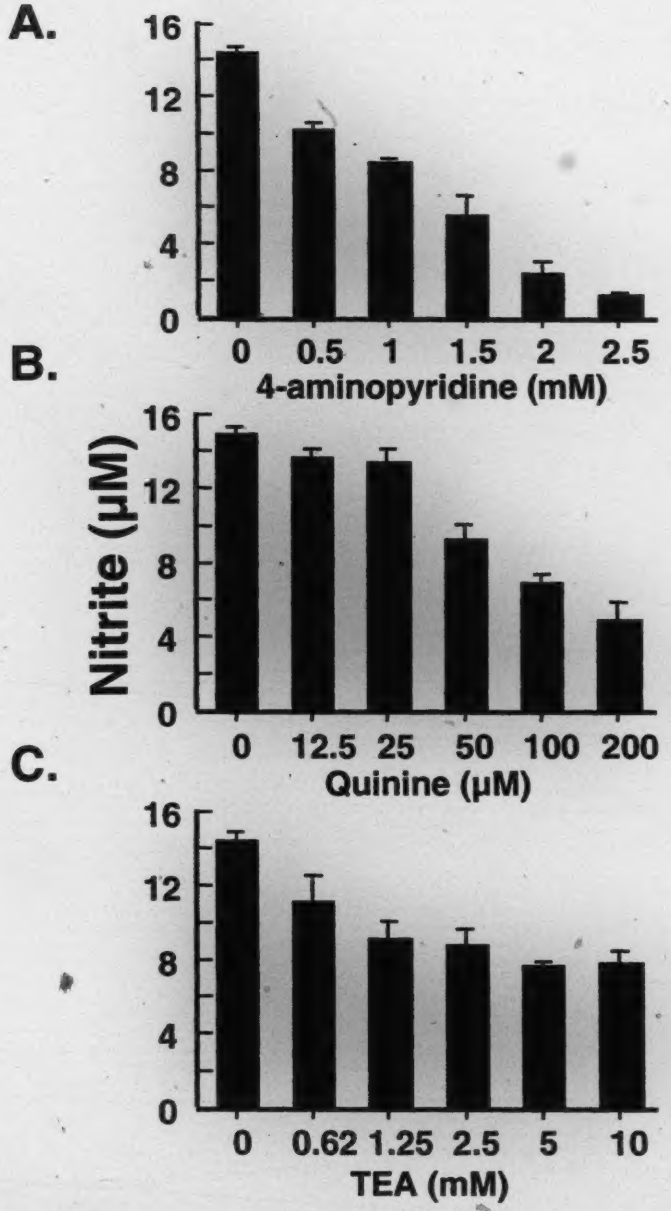

Figure 4.13. Dose-response inhibition of NO production by potassium channel blockers. IVDKM (5 x 10^4/well) were treated with varying doses of the potassium channel blockers 4-AP (A), quinine (B), and TEA (C). Nitrite production was determined in each treatment group after 72 h incubation at 72°C using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and the data are from a representative experiment from three independent experiments performed.

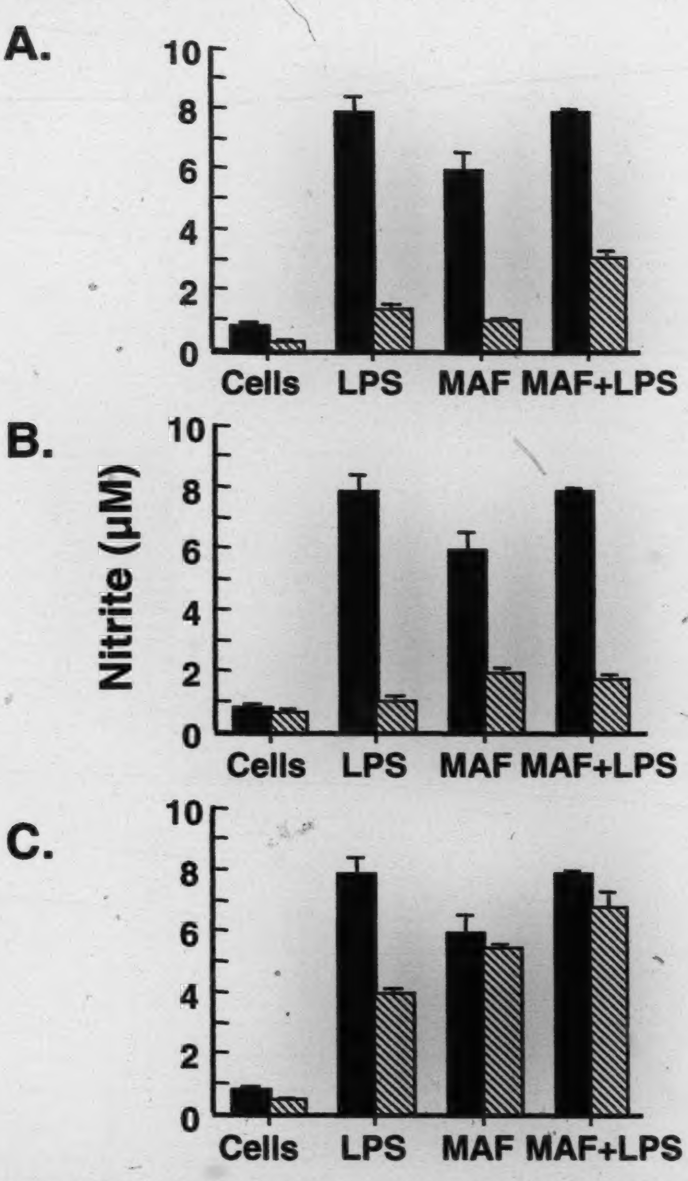

Figure 4.14. The effect of potassium channel blocking on NO production by macrophages activated with LPS and/or MAF. IVDKM (5 x 10^4/well) were treated with 2.5 mM, 200 µM, and 10 mM of 4-AP (A), quinine (B), and TEA (C), respectively. Cells were simultaneously activated with LPS (10 µg/ml), MAF (1:4), or MAF (1:4) and LPS (1 µg/ml) and NO production determined after 72 h incubation at 20°C using the Griess reaction. Solid bars represent cells without potassium channel blockers and hatched bars represent cells treated with potassium channel blockers. A control group indicated as 'cells' were treated with medium alone or medium supplemented with the indicated potassium channel blocker. Each bar represents the mean ± SEM of triplicate cultures and the data are from a representative experiment from three independent experiments performed.

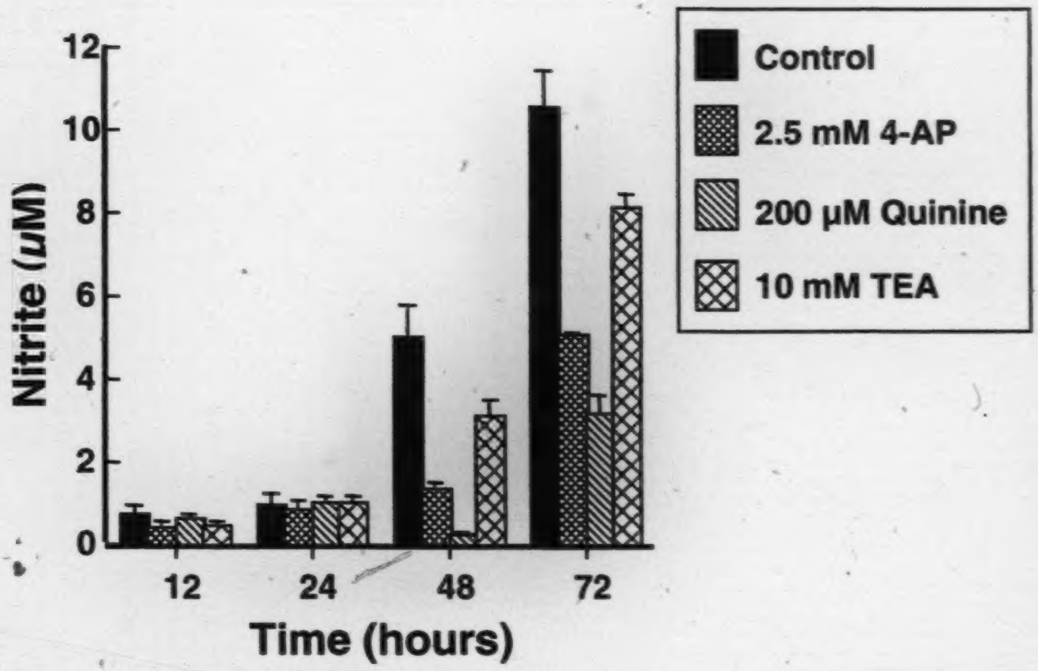

Figure 4.15. Time-course of NO production by goldfish macrophages in the presence of potassium channel blockers. IVDKM (5 x 10^4/well) were treated with 2.5 mM, 200 µM, and 10 mM of 4-AP, quinine, and TEA, respectively. Cells were simultaneously activated with LPS (10 µg/ml) and supernatants removed after 12, 24, 48, and 72 h incubation at 20°C and analyzed for NO production using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and the data are from a representative experiment from three independent experiments that were performed.

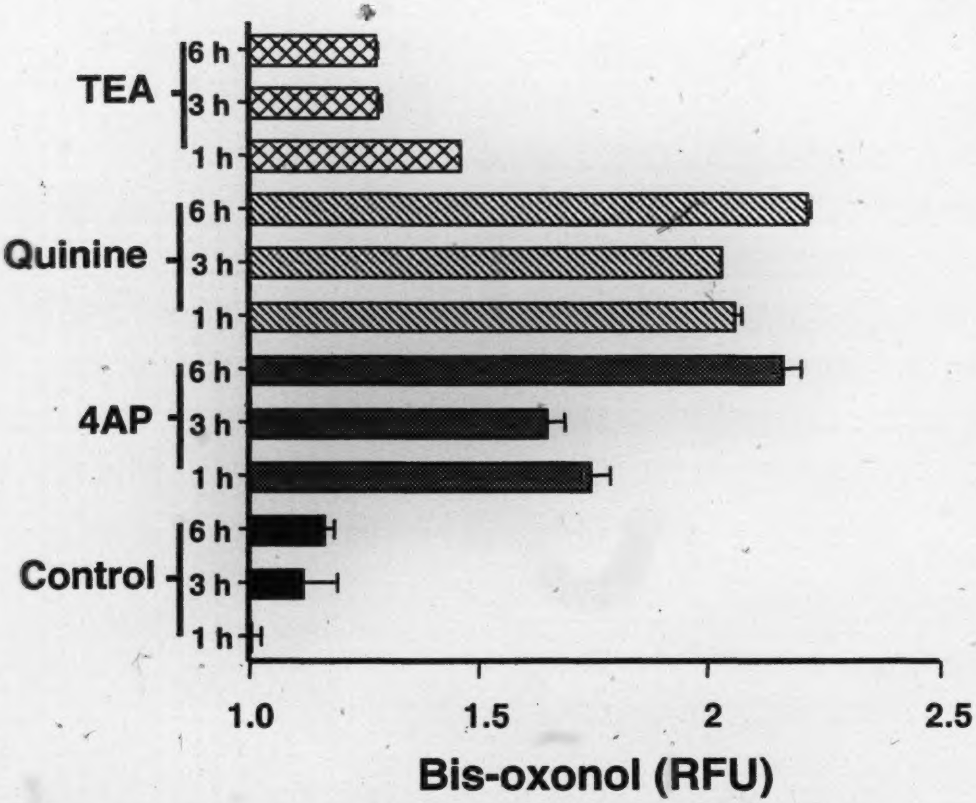

Figure 4.16. Modulation of goldfish macrophage membrane potential (V_m) by treatment with potassium channel blockers. IVDKM were seeded into polypropylene tubes (2.5×10^5 cells/tube) and treated with medium alone (control), 2.5 mM 4-AP, 200 μM quinine, or 10 mM TEA. After the indicated incubation times, cells were stained for 25 min with 5 μM bis-oxonol. Bis-oxonol was removed by washing 2x with medium alone or medium supplemented with the appropriate potassium channel blocker. Fifty μl of each treatment (5×10^4 cells) were then plated in triplicate, and RFU determined on a fluorimeter. Due to variable loading between experiments, fluorescence values were normalized to the RFU of 1 h control cells by dividing RFU of treated cells by RFU of 1 h control cells. Following normalization of RFU values, control macrophage RFU=1.0. See results section for significance of changes in bis-oxonol fluorescence intensity. Each bar represents the mean ± SEM of triplicate cultures and the data are from a representative experiment from three independent experiments that were performed.

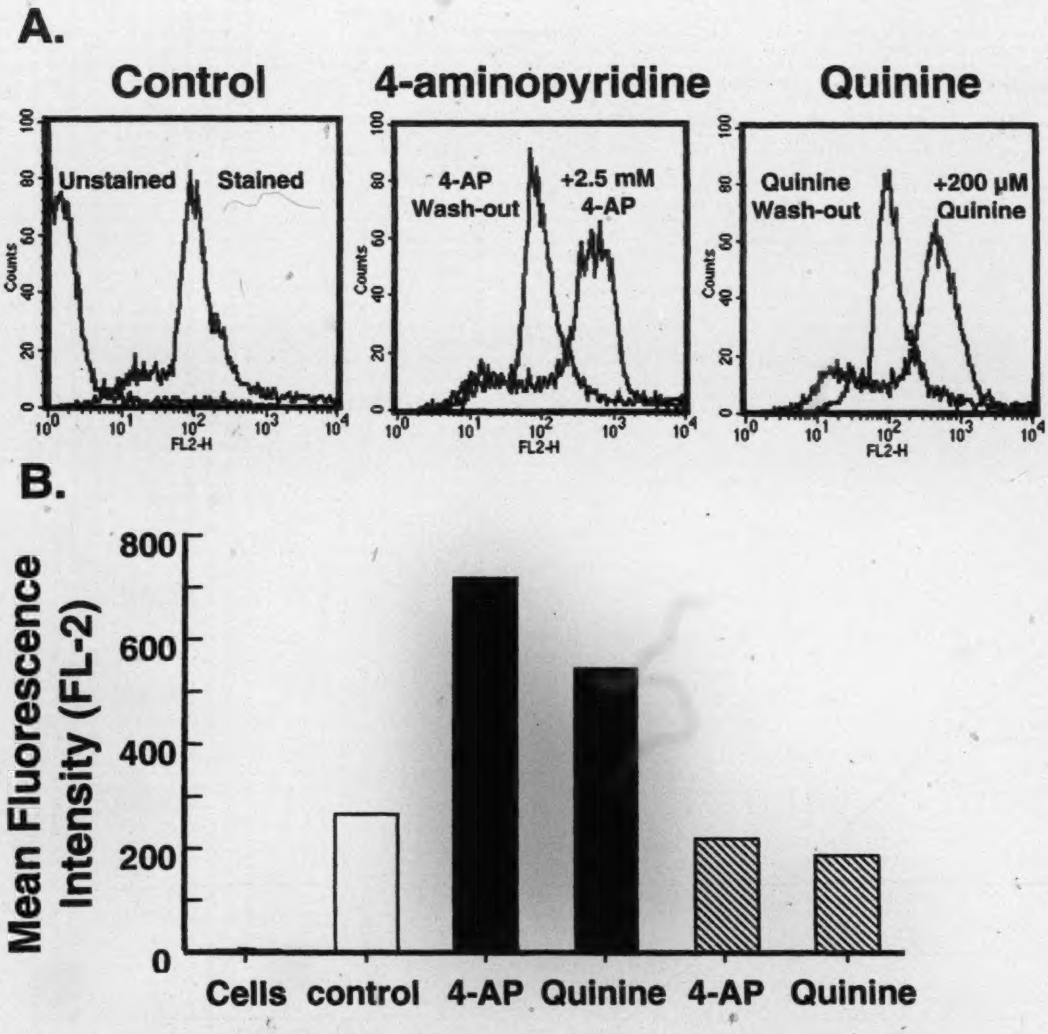

Figure 4.17. Reversibility of V_m changes by removal of potassium channel blockers. IVDKM were seeded into polypropylene tubes (2.5 x 10^5 cells/tube) and treating with medium (control; white bar), 2.5 mM 4-AP, or 200 µM quinine (black bars) for 3 h at 20°C. Following incubation, cells were washed 2x with medium alone (control and wash-out group) or medium supplemented with potassium channel blocker. After washing, cells were loaded for 25 min with 5 µM bis-oxonol. Residual bis-oxonol was removed by washing the cells 2x and RFU's determined by flow cytometry. Bis-oxonol loading was confirmed by the control group, which shows a shift in FL-2 fluorescence following staining (A). Treatment with 2.5 mM 4-AP or 200 µM caused significant shifts in FL-2 fluorescence above stained control cells (i.e. depolarization). This shift of fluorescence was reversed after washing-out the potassium channel blockers (hatched bars). The mean fluorescence intensities (FL-2) are summarized (B) and each bar represents the mean of duplicate cultures. Data are from a representative experiment from three independent experiments performed.

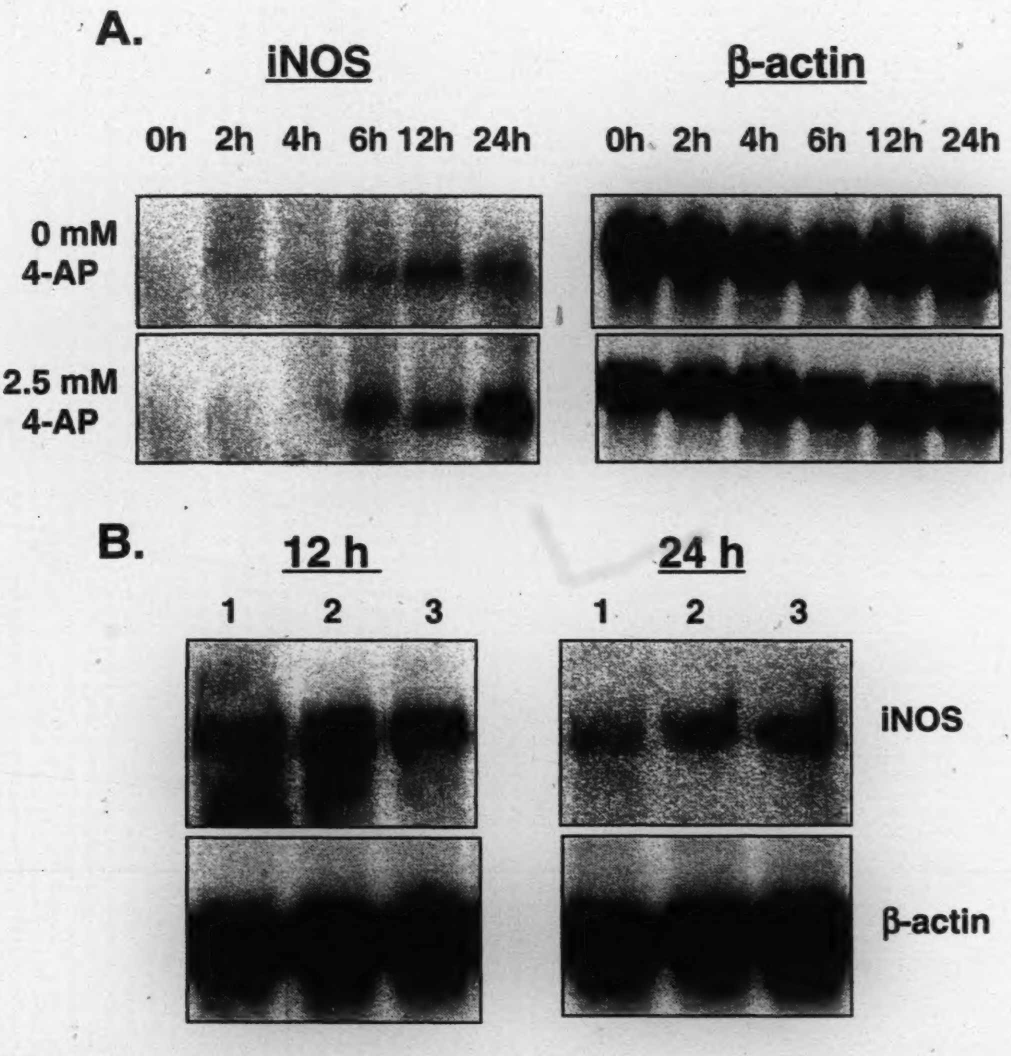

Figure 4.18. Changes in the expression of iNOS mRNA in the presence of potassium channel blockers. Northern blot analysis of iNOS mRNA by control cells (0 mM 4-AP) and by potassium channel treated cells (2.5 mM) after 0, 2, 4, 6, 12, and 24 h following activation with 10 µg/ml LPS (A). Quinine and TEA were also tested for their effects on goldfish macrophage iNOS expression after 12 and 24 h activation with 10 µg/ml LPS (B). Lane 1=control cells, Lane 2=10 mM TEA treated cells, and Lane 3=200 µM quinine treated cells. Data are from a representative experiment of two independent experiments that were performed.

CHAPTER 5

BIOCHEMICAL AND FUNCTIONALCHARACTERIZATION OF A MACROPHAGE ACTIVATION FACTOR THAT INDUCED NITRIC OXIDE PRODUCTION IN GOLDFISH MACROPHAGES[4]

5.1 Introduction

It is well established that mitogen-stimulated mammalian leukocytes secrete a number of soluble factors responsible for mediating macrophage antimicrobial functions [617-618]. These factors, or cytokines, have been extensively characterized based on functional and biochemical properties. Among these cytokines, IFNγ is the primary macrophage activation factor (MAF) found in the mitogen-stimulated leukocyte supernatants [342, 619]. The induction of NO in mammalian macrophages occurs as a result of stimulation with cytokines (e.g. IFNγ) and/or bacterial or parasite-derived factors (e.g. LPS) [406]. Macrophage-derived NO has been shown to play a major role in the cellular defence against invading pathogens, such as bacteria, protozaons, helminths, and tumors [395, 406].

Supernatants derived from mitogen-stimulated goldfish kidney leukocytes, have also been shown to synergize with LPS for the induction of NO in goldfish macrophages [318]. However, the native factor(s) that mediated this response have not beeen identified. In chapter 4, I reported that two major activities were present in partially-purified mitogen-stimulated goldfish kidney leukocyte supernatants. These factors exhibited both NO-inducing (MAF) and NO-inhibiting (MDF) activities, which suggested that the control of goldfish macrophage antimicrobial functions (i.e. activation and deactivation) is regulated through the concerted actions of multiple factors present in these supernatants [322-323]. Based on the preliminary biochemical and functional characterization, the leukocyte-derived MDF may be the telesot equivalent of TGFβ (Chapter 4), but the precise molecular identity of a native fish MDF or a native fish macrophage NO-inducing factor(s) remained to be identified.

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[4] A version of this chapter has been published: Stafford *et al.*, 2001. *Dev. Comp. Immunol.*, 25: 101-115; and Stafford and Belosevic, 2003. *Dev. Comp. Immunol.*, 27: 539-554.

In this chapter, I described the results of experiments designed to characterize and identify a goldfish macrophage NO-inducing factor that was purified from mitogen-stimulated goldfish kidney leukocyte supernatants. To date, the identification of teleost 'cytokine-like' molecules has primarily depended upon degenerate PCR and molecular cloning techniques [357-358, 367, 369, 589-597]. Until recently, no information was available regarding the functional properties of these cloned factors, however, with the recent production of recombinant fish proteins, some preliminary functional data are now available [363, 381, 597]. Investigators have also attempted to identify native fish MAFs, but have only referred to these factors as 'cytokine-like' based on functional and biochemical properties. For example, a heat and acid labile, T-cell-derived MAF, that exhibited activities similar to mammalian IFNγ, has been reported for rainbow trout [314]. In addition to this IFNγ-like factor, there appear to be other MAFs present in mitogen-stimulated leukocyte supernatants. Lipopolysacharride combined with turbot leukocyte supernatants, was shown to be capable of inducing turbot macrophages to produce NO, and the same response was observed when LPS was combined with turbot IFNα/β-like molecules [325]. Human TNFα was shown to augment the respiratory burst response of rainbow trout macrophages, and monoclonal antibodies to the high affinity hTNFα receptor impaired this response [355]. In turbot, the combination of LPS with hTNFα significantly increased the production of NO in turbot macrophages [325]. These findings suggest that fish produce both IFN- and TNFα-like molecules, which has been recently confirmed by molecular cloning in different fish species [357-358, 595-597].

In an effort to identify the factors that mediate goldfish macrophage antimicrobial functions, we have attempted to clone various goldfish 'cytokine-like' genes using degenerate PCR and have recently screened a goldfish kidney leukocyte library. We have also tested a panel of commercially available polyclonal and monoclonal anti-mammalian cytokine antibodies that are known to modulate macrophage antimicrobial functions (e.g. IL-2, IL-1, TNFα, IFNγ, and M-CSF). In our laboratory, these approaches have failed to identify a putative goldfish factor with macrophage activating properties. As such, I have chosen to characterize a goldfish macrophage NO-inducing factor using the more traditional biochemical and functional approaches.

The findings presented in this chapter demonstrate that in addition to cytokine-like factors, there are other proteins present in mitogen-stimulated leukocyte supernatants that can modulate fish macrophage antimicrobial functions. Specifically, I report on the biochemical and functional chacracterization of a goldfish macrophage NO-inducing factor as well as the identification of this native molecule as truncated forms of the iron-binding serum protein, transferrin.

5.2 Results

5.2.1 Detection of goldfish macrophage NO-inducing factor activity in mitogen-stimulated goldfish kidney leukocyte supernatants

As descriped in chapter 4, mitogen-stimulated goldfish kidney leukocyte supernatants were separated by GP-FPLC and two major biological activities were observed. Factors with NO-inducing (MAF) and NO-inhibiting (MDF) activities were present in these supernatants (see Fig. 4.1). The NO-inducing factor was estimated to be ~50 kD based on GP-FPLC size standards. In addition, a 30-35 kD MAF has also been described, which induced the production of ROI by goldfish macrophages [322] [334]. Preliminary biochemical characterization of a goldfish macrophage NO-inducing factor was used for the partial purification of this molecule(s) and the generation of a polyclonal anti-NO-inducing factor antibody [478]. This antibody was then used to identify the leukocyte-derived factor(s) that induced the production of NO by stimulated goldfish macrophages as described below.

5.2.2 Western blot analysis of crude and FPLC-separated mitogen-stimulated goldfish kidney leukocyte supernatants

A polyclonal antibody, designated as 8C2, was raised by immunizing rabbits with an FPLC-purified NO-inducing factor (Fig. 5.1A, lane 3). Western blot analysis of crude mitogen-stimulated goldfish leukocyte supernatants, using the anti-NO-inducing factor antibody as a probe, resulted in the recognition of 5 distinct protein bands (doublet at ~55-60 kD), and three other proteins with approximate MW of ~33 kD, ~35 kD and ~37 kD (Fig. 5.1B). These five protein bands were recognized by the anti-NO-inducing factor antibody in crude leukocyte supernantants, in the GP-FPLC fraction with maximal activity, and in the C-FPLC-purified NO-inducing factor fraction (Fig 5.1A, Lanes 1, 2, and 3, respectively).

5.2.2.1 Time-course analysis of mitogen-stimulated leukocyte supernatant production

Out of the 5 proteins recognized by the anti-NO-inducing factor antibody, the three smaller proteins (~33 kD, ~35 kD, and ~37 kD) correlated with the ability of leukocyte supernatants to induce NO production at 24, 48, and 72 h (Fig. 5.2A). At 0 h, these proteins were not detected by the anti NO-inducing factor antibody and the production of NO by goldfish macrophages was not observed. After 24 h, supernatants from goldfish kidney leukocytes stimulated by MLR and/or mitogens, contained the ~33 kD, ~35 kD, and ~37 kD proteins, and also exhibited NO-inducing factor activity (Fig. 5.2). A control group of unstimulated goldfish kidney leukocytes (i.e. no MLR or mitogens) was included to demonstrate that the appearance of these three proteins was dependent on the presence of stimulated kidney leukocytes. This control was also included to demonstrate that residual mitogens, potentially remaining after the wash-out step, were not present in sufficient amounts to induce the production of NO by goldfish macrophages.

5.2.2.2 Western blot detection of NO-inducing factor in GP-FPLC separated mitogen-stimulated leukocyte supernatants; correlation of ~33-37 kD proteins with NO production

Prior to FPLC purification, crude leukocyte supernatants were concentrated (~30-fold), re-tested for NO-inducing activity, and examined by Western blot (Fig. 5.3). Concentrated kidney leukocyte supernatants retained NO-inducing activity, however, at the lowest dilution tested (i.e. 1/4), decreased levels of NO production were observed than compared to the higher dilutions (i.e. 1/8 to 1/32; Fig. 5.3B). Conversely, non-concentrated leukocyte supernatants induced the highest production of NO at the lowest dilution tested (i.e. 1/4), with lower activities observed with increasing dilutions of the supernatant. This suggests that there is a dose-dependent induction of NO production by this factor(s). At higher concentrations, the NO-inducing factor may be toxic to goldfish macrophages causing the observed reductions of NO producton. However, this is one possibility and the viability of goldfish macrophages treated with concentrated supernatants was not determined.

During the concentration procedure, a visible precipitate was formed. To ensure that proteins with NO-inducing activity were not present in this precipitate (and lost as a result of the concentration procedure), these proteins were re-dissolved in 1xPBS at a volume equivalent to the non-concentrated original sample. At all dilutions tested, the re-dissolved proteins failed to induce NO in goldfish macrophages and no proteins were detected by Western blot using the anti-NO-inducing factor antibody (Fig. 5.3A).

Concentrated mitogen-stimulated leukocyte supernatants were then separated by molecular size using GP-FPLC. Superose 12 fraction #28 demonstrated maximal NO inducing activity, which eluted to an approximate molecular weight of ~50 kD (Fig. 5.4). Western blot analysis of the fractions that exhibited maximal NO-inducing activity using the anti-NO-inducing factor antibody as a probe, demonstrated the presence of all 5 protein bands initially recognized in the crude mitogen-stimulated leukocyte supernatants (Fig 5.4A). However, the ~33 kD, ~35 kD, and ~37 kD proteins correlated more precisely with induction of NO production by goldfish macrophages, whereas the ~55-60 kD protein did not.

5.2.2.3 Western blot detection of NO-inducing factor in ion-exchange separated mitogen-stimulated leukocyte supernatants; correlation of ~33-37 kD proteins with NO production

Ion-exchange chromatography was chosen to further purify the NO-inducing factor since under the conditions chosen, this factor bound to the Mono-Q column enabling multiple injections of the sample possible (Fig. 5.5). Twenty injections of concentrated mitogen-induced leukocyte supernatants (or pooled GP-FPLC NO-inducing factor fractions #27 and #28) were injected onto a Mono-Q column and both the bound and unbound proteins tested for activity. Proteins that did not bind the Mono-Q column (i.e. fractions #1-10) consistently failed to induce production of NO (data not shown) in goldfish macrophages. However, following elution of bound proteins from the column using an increasing NaCl gradient [indicated by diagonal line (inset Fig 5.5C)], maximal NO-inducing activity was consistently observed in fractions #14-16. SDS-PAGE and Western blot analysis using the anti-NO-inducing factor antibody, demonstrated that proteins with the approximate MW of 33, 35 and 37 kD induced maximal NO activity (Mono-Q fraction #15; Fig. 5.5A and Fig. 5.5B).

5.2.3 Evidence that the ~33 kD, ~35 kD, and~37 kD putative NO-inducing factors are tranferrin cleavage products

5.2.3.1 Mass Spectrometry analysis of putative goldfish macrophage NO-inducing factor

Following Mono-Q purification, the ~33 kD, ~35 kD, and ~37 kD putative NO-inducing factor proteins bands were cut from a Coomassie-blue stained SDS-PAGE gel and analyzed by mass-spectrometry. All three proteins were subsequently identified as serotransferrin precursor (Siderophilin; GenBank Accession #U02564) and the sequences of the peptide fragments are shown (Table 5.1). The larger ~55-60 kD protein that was also identified by the anti-NO-inducing factor antibody protein was also previously identified as serotransferrin precursor (data not shown). In addition, all fragments had the highest amino acid homology with bovine transferrin. The sequences of the individual peptide fragments identified by mass-spectrometry were then aligned with the amino acid sequence of GenBank accession #U02564 (Fig. 5.6). All peptide fragments analyzed were found to share 100% amino acid homology with various regions of the bovine transferrin sequence (letters in bold denote peptide fragments identified by mass-spectrometry; Fig. 5.6). From the analysis of the individual peptide fragments, it was possible to interpret that the smaller protein (33 kD), represented the N-lobe of transferrin. All but one of the peptide fragments of this protein identified by mass-spectrometry aligned prior to the peptide bridge region (underlined) that separates the N- and C-lobes of native transferrin (only one peptide fragment, CLVEKGDVAFVK, was found after the peptide bridge for the 33 kD protein and may have resulted from cross-contamination with the other proteins). The other two proteins (35 kD and 37 kD) appeared to be identical since they contained 7 identical peptide fragments (Table 5.1). In addition, this protein appears to be the C-lobe of transferrin since all the peptide fragments were identified after the peptide bridge region with the exception of the peptide fragment GDVAFVK, which was identified on both sides of the bridge peptide (Fig. 5.6).

5.2.3.2 Protease-digestion and FPLC purification of bovine transferrin

Since mass-spectometry analysis identified that the NO-inducing factor purified from goldfish kidney leukocyte supernatants was transferrin, which exhibited the highest amino acid homology with bovine transferrin, commercially available bovine transferrin (Sigma) was separated by SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with the anti-NO-inducing factor antibody (Fig. 5.7). The anti NO-inducing factor antibody recognized 1 μg of bovine transferrin at 1/100 to 1/10 000 dilution, confirming that the polyclonal NO-inducing factor antibody, which was originally generated from the FPLC purified NO-inducing factor, was indeed recognizing bovine transferrin. Furthermore, bovine transferrin was protease-digested *in vitro* to determine if cleavage of this protein resulted in the generation of fragments with approximate MW of 33-37 kD, as originally identified in the crude and FPLC-separated leukocyte supernatants. The protease-digested transferrin fragments were then fractionated by FPLC using identical procedures for the initial purification of the NO-inducing factor from mitogen-stimulated goldfish kidney leukocyte supernatants. Protease-digestion of bovine transferrin resulted in the cleavage of the precursor protein (~55-60 kD) into truncated forms consisting of three protein bands with approximate MW of 33 kD to 37 kD (Fig. 5.8A). The anti-NO-inducing factor antibody generated against FPLC-purified mitogen-stimulated goldfish leukocyte supernatants also recognized the digested forms of protease-digested bovine transferrin (Fig. 5.8A). The digested proteins were subsequently fractionated by GP-FPLC using a Superose 12 column (Fig. 5.8B) and once again were analyzed by SDS-PAGE and Western blot (Fig. 5.8C). As expected, the transferrin fragments eluted to the identical fractions as did the NO-inducing factor originally purified from mitogen-stimulated leukocyte supernatants (compare Fig. 5.8C and Fig. 5.4A).

GP-FPLC separated bovine transferrin fragments were then separated by ion-exchange chromatography using identical procedures for the Mono-Q purification of the goldfish leukocyte-derived NO-inducing factor (Fig. 5.9A). The cleaved transferrin fragments eluted to the identical fractions as did the Mono-Q purified NO-inducing factor (i.e. Mono-Q fractions #14-17; compare Fig. 5.9 and Fig. 5.5). Interestingly, a small but significant ($P<0.0001$) induction of NO production by Mono-Q separated bovine transferrin fragments was observed for fraction #14, which closely correlates with the active fraction from Mono-Q purified NO-inducing factor from goldfish mitogen-stimulated leukocyte supernatants. This further supported the observation that transferrin cleavage products function as NO-inducing factors in goldfish macrophages.

5.2.3.3 Enhancement of the NO-inducing activities of mitogen-stimulated goldfish kidney leukocyte supernatants by the addition of bovine transferrin

Mitogen-stimulated leukocyte supernatants were generated after an overnight incubation with 5% carp serum only (i.e. no bovine serum). Following a 6 h stimulation period, the mitogens and serum were removed and the leukocytes were incubated in serum-free medium (control) or in serum-free medium supplemented with 25 μg/ml of undigested bovine transferrin. Transferrin was added to determine: a) whether mitogen-stimulated goldfish leukocyte supernatant components can cleave bovine transferrin into the ~33 kD, ~35 kD, and ~37 kD truncated forms; and b) what effect does the addition of bovine transferrin have on the NO-inducing potential of mitogen-stimulated leukocyte supernatants. The addition of bovine transferrin to mitogen-stimulated leukocyte cultures resulted in the cleavage of the native protein (~55-60 kD), into truncated forms with approximate MW of 33, 35, and 37 kD (Fig. 5.10A). Time-course analysis of these supernatants using Western blot revealed that the digested forms of transferrin were absent at 0 h, initially appeared at 24 h, and increased in intensity by 72 h (Fig. 5.10A). Sub-samples removed at the various time-points were also tested for NO-inducing activity, which revealed that in the presence of bovine transferrin, the NO-inducing potential of the mitogen-stimulated leukocyte supernatants was significantly enhanced (Fig. 5.10B).

5.2.4 Evidence that fish transferrin is responsible for the NO-inducing activity present in mitogen-stimualted goldfish kidney leukocyte supernatants

5.2.4.1 NO-inducing activity of mitogen-stimulated goldfish kidney leukocyte supernatants was observed in the absence of bovine transferrin

A common procedure used by many laboratories was to incubate mitogen-stimulated fish leukocytes in the presence of bovine serum often in the absence of any fish serum [314-315, 317-318, 322, 325, 328, 620]. To determine whether the NO-inducing activities present in these preparations can be modulated by 'fish-derived' factors only, mitogen-stimulated goldfish leukocyte supernatants were prepared under the following conditions: overnight incubation in; a) 10% FCS and 2.5% carp serum; b) 10% FCS alone; or c) 5% carp serum only. The supernatants were then tested for NO-inducing activity, and as shown in Figure 5.11, the NO-inducing activity of stimulated goldfish leukocyte supernatants was not dependent on the presence of bovine serum components, indicating that 'fish-derived' factors alone can induce fish macrophage NO production.

5.2.4.2 Purification of fish transferrin and detection of truncated forms of this protein in FPLC fractions with NO-inducing factor activity

To determine whether fish transferrin was responsible for modulating goldfish macrophage function, transferrin was purified from the serum of carp (carp serum was chosen since it was initially used in preparation of the leukocyte supernatants). Using a modified procedure for the purification of human serum transferrin, carp serum transferrin was subjected to DEAE Affi-Gel-Blue chromatography, Con A affinity chromatography, and ion-exchange chromatography (Fig. 5.12). Carp serum was applied to the DEAE column after dialysis against 20 mM Tris-HCl, 25 mM

NaCl, pH 9.3, which resulted in the optimal recovery of transferrin with fewer other serum protein contaminants (Fig. 5.12, Lane 1 (arrow)). Proteins were then separated by Con A chromatography, and in contrast to bovine transferrin, the majority of carp serum transferrin was present in the unbound fraction, indicating that the majority of carp serum transferrin was non-glycosylated (Fig. 5.12B, compare lanes 2 and 3). Con A-purified carp serum transferrin was then protease-digested as described for the digestion of bovine transferrin, (Fig. 5.12, lane 4) and the fragments separated by ion-exchange chromatography (Mono-Q) to obtain a purified fraction of carp serum transferrin cleavage products (Fig. 5.12, lane 5). Purified cleavage products were then used to immunize rabbits for the generation of a polyclonal anti-carp serum transferrin antibody. Designated as 9AG7, this antibody recognized 1 μg of carp serum transferrin cleavage products at dilutions ranging from 1/100 to 1/10 000 (Fig. 5.13).

5.2.4.3 Cross-reactivity of anti-NO-inducing factor and anti-carp serum transferrin polyclonal antibodies and detection of the truncated forms of transferrin in fractions exhibiting maximal NO-inducing activity

Anti-carp serum transferrin antibody (9AG7) was generated to determine whether fish transferrin is responsible for the NO-inducing activity present in mitogen-stimulated goldfish kidney leukocyte supernatants. As shown in Figure 5.14, this polyclonal antibody recognized proteolytically-digested forms of fish transferrin in the various FPLC fraction that induced maximal NO production in goldfish macrophages. Furthermore, the data clearly showed that the two polyclonal antibodies (i.e. 8C2 and 9AG7) did not cross-react and recognize epitopes of transferrin specific for each species (compare Fig. 5.14A with Fig. 5.14B). Interestingly, the carp serum transferrin polyclonal antibody also recognized goldfish transferrin (Fig. 5.16 and Fig. 5.17).

5.2.4.4 Detection of fish transferrin fragments in the active fractions of FPLC-separated leukocyte supernatants prepared with fish serum only

GP-FPLC separation of mitogen-stimulated goldfish leukocyte supernatants, after an overnight incubation with 5% carp or goldfish serum only, resolved maximal NO-inducing activity in the fractions where the majority of truncated fish transferrin fragments were shown to elute (i.e. Superose 12 fractions #28, 29, and 30; Fig. 5.15). Following C-FPLC (Mono-P) or ion-exhange chromatography (Mono-Q) of leukocyte supernatants prepared in the presence of fish serum, maximal NO-inducing factor activity was exhibited by fraction #3 and fractions #13-16, respectively. These were also the same fractions containing immuno-reactive proteins when incubated with the anti-carp serum transferrin antibody (9AG7) (Fig. 5.16 and Fig. 5.17).

5.2.5 Immunopurification and mass-spectrometry analysis of goldfish transferrin cleavage products that exhibit NO-inducing factor activity

5.2.5.1 Immunopurification of transferrin from goldfish serum

The polyclonal anti-carp serum transferrin IgG was purified and ligated to a 1 ml HiTrap NHS-activated HP column purchased from Amersham Pharmacia. To ensure that the purified antibody was capable of recognizing/binding goldfish transferrin, a sample of DEAE partially-purifed goldfish serum was applied to the column and the bound and unbound proteins analysed by SDS-PAGE and Western blot (Fig. 5.18). DEAE separated goldfish serum contained a variety of proteins as detected by silver-staining (Lane 1, Fig. 5.18). However, after passing through the immuno-affinity column, only one distinct protein band was detected, which was also recognized by the anti-carp serum transferrin polyclonal antibody (Fig. 5.18, lanes 2 and 3, respectively). These findings indicate that the purified IgG bound to the NHS-activated HP column was capable of recognizing and binding golfish serum transferrin in its 'native' form.

5.2.5.2 Immunopurification of NO-inducing factor from mitogen-stimulated goldfish kidney leukocyte supernatants

I have shown that proteolytic cleavage products derived from both bovine and fish transferrin induced NO response of goldfish macrophages. To confirm these findings I developed an immunoaffinity procedure using the anti-carp transferrin polyclonal antibody, which also recognized goldfish transferrin. Mitogen-stimulated goldfish kidney leukocyte supernatants were prepared in the presence of goldfish serum only and partially purified using ionic exchange chromatography. The Mono-Q fractions that contained maximal NO-inducing activity (fractions #13-16) also contained transferrin cleavage products (Fig. 5.17). These fractions were pooled and applied to the immunoaffinity column. As shown in Fig. 5.19, proteins that bound to the immunoaffinity column correlated to the approximate molecular weights of native transferrin (~55 kD) and the cleavage products (~ 33kD, ~35 kD, and ~37 kD) as previously described. Protein bands detected in the bound fraction were also recognized by the anti-carp serum transferrin polyclonal antibody following Western blot analysis (Fig 5.19A, lane 4). Treatment of goldfish macrophages with the immunoaffinity-purified fraction demonstrated that the proteins recognized by the anti-carp transferrin antibody (i.e. bound to the immunoaffinity column) also exhibited NO-inducing factor activity (Fig 5.19B). Relatively low levels of NO were induced by the unbound fractions, compared with the pre-affinity (pooled Mono-Q fractions #13-16) or bound fractions, however, there was a significant difference in the amount of nitrate induced by the bound immunoaffinity fraction (P<0.05).

5.2.5.3 Mass-spectrometry analysis of immunopurified goldfish macrophage NO-inducing factor

Immunopurified goldfish macrophage NO-inducing factor was analyzed at the UVic-Genome BC Proteomics Centre located on the University of Victoria campus. MALDI-TOF analysis identified proteins with molecular masses of approximately 71 455, 37 294, 35 788, and 33 993 kDa that correlated to the protein bands (#1, 2, 3, and 4; Fig. 5.19A) identified by Western blot of the bound fraction following immunopurification. In addition, all 4 of these proteins were identified as goldfish transferrin using tryptic digest mapping analysis (data not shown). Based on the structure of the transferrin molecule, it was hypothesized that the native protein (~71 455 kD) was cleaved somewhere between the N-lobe (~33 993 kD) and C-lobe (~35 788 kD and ~37 294 kD). Presence of two protein bands representing the C-lobe is most likely due to glycosylation sites identified in the C-lobe but not the N-lobe of the tansferrin protein [487]. No other proteins were identified in the immunopurified fraction.

5.3 Discussion

In an attempt to purify and characterize a native NO-inducing factor produced by mitogen-stimulated goldfish kidney leukocytes, I unexpectedly identified that cleaved fragments of transferrin are important regulators of the macrophage NO response in goldfish. Proteins with NO-inducing activities were purified from the supernatants of mitogen-stimulated goldfish kidney leukocyte cultures using gel permeation, ion exchange, and chromatofocusing FPLC. In addition, immunopurification was performed to confirm that fish transferrin induced the production of NO in goldfish macrophages. The isolated proteins that exhibited NO-inducing factor activity were then analyzed using mass spectrometry. The putative NO-inducing factor was identified as truncated forms of the serum protein transferrin, and the active fragments had approximate MW of 33, 35, and 37 kD. The native form (i.e. full-length) of transferrin did not enhance NO production in LPS-stimulated goldfish macrophages, but enzymatic cleavage of this protein correlated with enhanced production of NO in goldfish macrophages.

The cleavage of transferrin was dependent on the presence of stimulated kidney leukocytes and was shown to occur in response to both MLR and mitogenic stimulation. Time-course analysis by Western blot, using an anti-NO-inducing factor polyclonal antibody, demonstrated that 24 h after MLR and/or mitogen stimulation, cleaved transferrin products appeared in the supernatants of cultured cells, and this correlated with the onset of NO-inducing activity of these preparations. To confirm that the cleavage of transferrin resulted in the release of these fragments, transferrin was digested *in vitro*. The resulting cleavage products also had approximate MW of

33, 35, and 37 kD, and were recognized by the anti-NO-inducing factor antibody. When these peptides were subjected to the purification protocols used to original purify the NO-inducing factor from goldfish leukocyte supernatants, they were shown to elute to identical FPLC fractions as the fractions that contained maximal NO-inducing activity.

The source of transferrin was undoubtedly from the cultivation of fish kidney leukocytes in 10% calf serum and 5% carp serum prior to, and during, stimulation with mitogens. Although serum was removed by extensive washing of the cultures with 1xHBSS, and the leukocytes subsequently cultured in serum-free medium, transferrin was found to adhere to tissue culture flasks and leach into the culture medium during incubation (data not shown). Enzymatic cleavage of this residual transferrin may explain the presence of cleaved forms of this protein in the leukocyte culture supernatants. It is also possible that transferrin may have been produced by the stimulated leukocytes and subsequently cleaved.

The ability of transferrin cleavage products to modulate goldfish macrophage antimicrobial functions is novel, and there are several potential hypotheses to explain the results of this chapter. Goldfish macropahges cultures represent a heterogeneous population of macrophages, comprised of mature macrophages, monocyte-like macrophages and early progenitors of macrophage development [321, 335-336]. Mature macrophages appear to be the cell-type responsible for NO production, eliciting a characteristic response when stimulated with LPS and crude leukocyte supernatants (i.e. relatively little production of NO when stimulated with either factor, but a synergistic induction when co-stimulated with both factors [322]). In mammals, transferrin has been shown to a key mediator in the differentiation of monocytes and neutrophils into more mature phenotypes (i.e. monocytes into tissue macrophages) [185-186, 493-494, 510, 556]. The ability of transferrin to up-regulate NO production in goldfish macrophage cultures may be a result of the induced differentiation of monocyte-like macrophages into more mature tissue macrophages. This differentiation step appears to be a necessary pre-requisite for acquiring the capacity to produce NO, which I have discussed in Chapter 3.

Interestingly, Neumann *et al.*, [336] demonstrated that supernatants from mitogen-stimulated goldfish leukocytes (which also contain transferrin cleavage products) induced the differentiation of sorted monocyte-like cells into a more mature macrophage phenotype. Although full-length transferrin failed to enhance NO production in goldfish macrophages, the differentiation of monocytes into mature macrophages may be dependent upon the recognition of the transferrin cleavage products that are released at the site of inflammation. Increased vascular permeability during an inflammatory reaction would permit the leakage of transferrin from the blood into the inflammatory site, a phenomenon that has been described in both mammals and fish [505-509]. The plethora of extracellular matrix proteases expressed during inflammation may contribute to the enzymatic cleavage of transferrin at this site. Thus, as monocytes infiltrate into the inflammatory locus, a receptor-mediated recognition of transferrin-derived peptides may initiate differentiation of infiltrating monocytes into the more mature tissue macrophage phenotype with increased NO-producing capability. Further work is required to establish a potential differentiation-mediated role for transferrin cleavage products.

Secondly, it is becoming increasingly evident that the immune system is acutely responsive to a variety of endogenous signals that are generated during injury and/or inflammatory processes. Many of these proteins are not produced by healthy cells but are made or modified by distressed or injured tissues. Examples of some of the recently identified endogenous ligands include fibrinogen [10], fibronectin [11], surfactant protein-A [152], heat-shock proteins [153-154], hyaluronan [155], and heparan sulfate [156]. All of these substances can activate macrophages and are believed to be recognized by a highly conserved family of innate immunity receptors known as Toll-like receptors (TLRs; reviewed in Chapter 1). We have also recently discovered a TLR homologue expressed by goldfish macrophages [160].

The recognition of endogenous 'danger' or 'alarm' signals (e.g. molecules produced by stressed cells, or products that are usually found inside a healthy cell) by the immune system is the basis of the 'danger' model, which proposed that cells of the immune system do not detect foreign material *per se* but rather immune cells (i.e. macrophages) detect cellular injury caused by microbial infection and other environmental stresses [621-622]. Recently, this model has been expanded into what is now called the 'surveillance' model of immune recognition. This model proposes that cells of the immune system recognize the degradation products of endogenous macromolecules, and also recognize molecules from necrotic cells and microorganisms [151]. Therefore, the degredation of the endogenous macromolecule, transferrin, into smaller fragments by enzymes released from mitogen-stimulated goldfish kidney leukocytes, may be an endogenous 'danger' signal capable of initiating the innate immune response in fish.

Two of the endogenous ligands known to activate mammalian macrophages are also found in the serum (e.g. fibrinogen and fibronectin). During inflammation, endothelial cell retraction induced by products released from damaged cells permits plasma constituents to escape the vasculature [623]. Extravasated plasma generates thrombin which proteolyses the endogenous protein fibrinogen, stimulating its deposition as fibrin [624]. Fibrinogen has recently been shown to be capable of inducing macrophage chemokine secretion, by a process that was shown to share similar signaling pathways to LPS-induced chemokine secretion in macrophages [10]. Another serum-derived protein, fibronectin, also induced genes encoding proinflammatory cytokines in macrophages and this response was also similar to cells treated with LPS [11]. Cellular fibronectins, which contain alternatively spliced exons encoding type III repeat extra domains, are produced in response to tissue injury [625] and appear to be endogenous activators of a variety of pro-inflammatory responses [626-629]. These findings demonstrate that during inflammation, the activation of immune cells can be mediated by modified host factors (i.e. serum proteins), and the induction of NO in goldfish macrophages by cleaved transferrin fragments may also be due to a similar pathway as fibrinogen and fibronectin induced immune activation.

Proteolytic cleavage of transferrin and the induction of macrophage activation by the cleaved fragments may also represent a primitive regulatory mechanism for the induction of NO. Iron transport proteins homologous to transferrin have been identified in several invertebrate species [488-490, 630], and increased production of this protein was observed following exposure of insect cells to bacteria. This suggested that invertebrate transferrins function as acute-phase proteins (i.e. are up-regulated during an immune response) [491, 630]. This has been similarily demonstrated for vertebrate transferrin [504, 631]. In addition to the conservation of transferrin-like molecules in organisms, the induction of macrophage activation by cleaved transferrin fragments may also be mediated by a highly conserved immune receptor. As previously mentioned, the recognition of modified host proteins and subsequent induction of immune responses are properties of a family of highly conserved innate immune receptors that were originally discovered in *Drosophilia* (i.e. Toll receptors) [632-633]. These receptors have received considerable attention as innate pattern recognition receptors (PRRs) that are not only important in the recognition of pathogen associated molecular patterns (PAMPS; e.g. LPS) but are important in initiation and transduction of the intracellular signals that induce innate immune mechanisms in macrophages [6]. Activation of TLRs by microbial ligands activates intracellular signaling events that lead to the activation of NF-κβ and MAPK cascades [119, 122-123, 127], which induces innate immune mechanisms such as the production of ROI, RNI and chemokine/cytokine secretion [6].

The activation of these receptors appears to also require the initiation of a protease cascade and subsequent cleavage and recognition of endogenous proteins [9-11, 634-636]. While the precise endogenous ligands/activators of a recently identifed goldfish macrophage TLR have not been identified, the induction of an innate immune response by transferrin cleavage products likely represents a highly conserved mechanism of immune activation. Future research is required to establish that transferrin cleavage products are indeed an endogenous ligand for fish and perhaps mammalian innate immune receptors such as TLRs.

Table 5.1 Mass-spectrometry identification of the 33, 35, and 37 kD putative NO-inducing factor(s) purified from mitogen-stimulated goldfish kidney leukocyte supernatants[1]

Protein	Peptide Fragments (Sequence)	Identity (GenBank Accession #)
33 kD	HSTVFDNLPNPEDRK DNPQTHYYAVAVVK DKPDNFQLFQSPHGK CLVEKGDVAFVK LYKELPDPQESIQR SVDDYQECYLAMVPSHAVVAR NYELLCGDNTR DSADGFLK TSHMDCIK KDTDFK	Serotransferrin precursor (Siderophilin) (Beta-1-Metal Binding Globulin) Accession #U02564
35 kD	GEADAMSLDGGYLYIAGK DQTVIQNTDGNNNEAWAK TSDANINWNNLK TAGWNIPMGLLYSK KTYDSYLGDDYVR GDVAFVK AMTNLR GYLAVAVVK FDEFFSAGCAPGSPR QQDDFGK	Serotransferrin precursor (Siderophilin) (Beta-1-Metal Binding Globulin) Accession #U02564
37 kD	GEADAMSLDGGYLYIAGK DQTVIQNTDGNNNEAWAK TSDANINWNNLK TAGWNIPMGLLYSK TYDSYLGDDYVR GDVAFVK AMTNLR GPNHAVVSR	Serotransferrin precursor (Siderophilin) (Beta-1-Metal Binding Globulin) Accession #U02564

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[1] Sequence analysis was performed at the Harvard Microchemistry Facility by microcapillary reverse-phase HPLC nano tandem mass spectrometry (µLC/MS/MS) on a Finnigan LCQ quadrupole ion trap mass spectrometer.

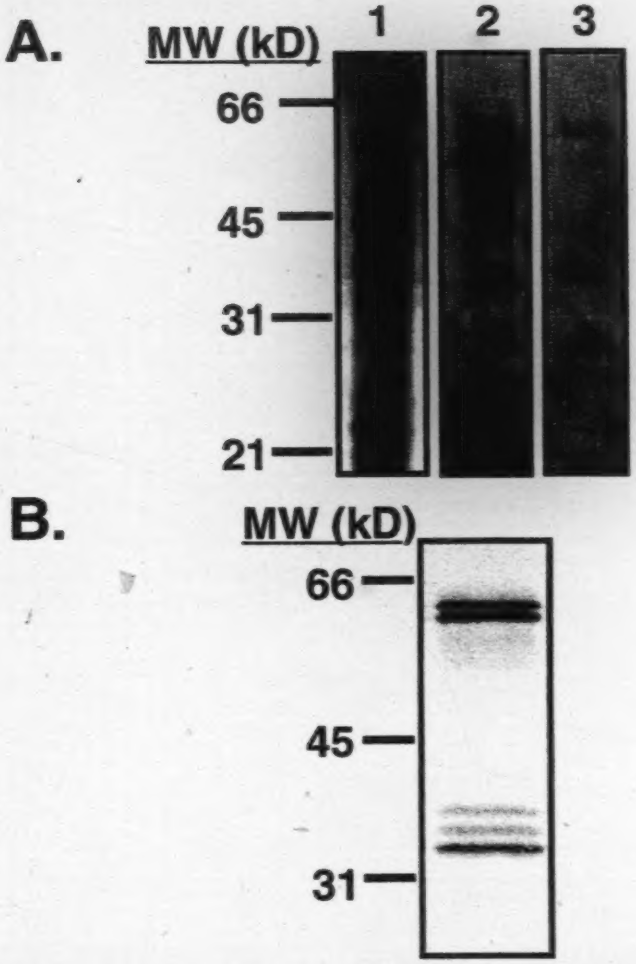

Figure 5.1. FPLC purification of a goldfish macrophage NO-inducing factor and production of an anti-NO-inducing factor polyclonal antibody. Mitogen-stimulated leukocyte supernatants were partially purified using GP-FPLC (Superose 12) and then fractionated by C-FPLC (Mono-P). Individual fractions from FPLC fractionated mitogen-stimulated leukocyte supernatants with maximal NO inducing activity were separated by denaturing SDS-PAGE and silver stained (A). Lane 1, crude leukocyte supernatant; Lane 2, GP-FPLC fraction #28 with maximal NO inducing activity; Lane 3, C-FPLC fraction with maximal NO-inducing activity. This fraction (lane 3) was used as the source of antigen for immunization of a rabbit. The anti-NO-inducing factor antibody (8C2) was then tested for ability to recognize proteins in crude and FPLC-separated mitogen-stimulated leukocyte supernatants (B). Western blot analysis of SDS-PAGE separated mitogen-stimulated leukocyte supernatants using 8C2 as the primary antibody at a 1/400 dilution. Note: the anti-NO-inducing factor antibody recognized 5 distinct protein bands in all samples analyzed (i.e. 2 bands approximately 55-60 kD, and ~33 kD, ~35 kD, and ~37 kD proteins).

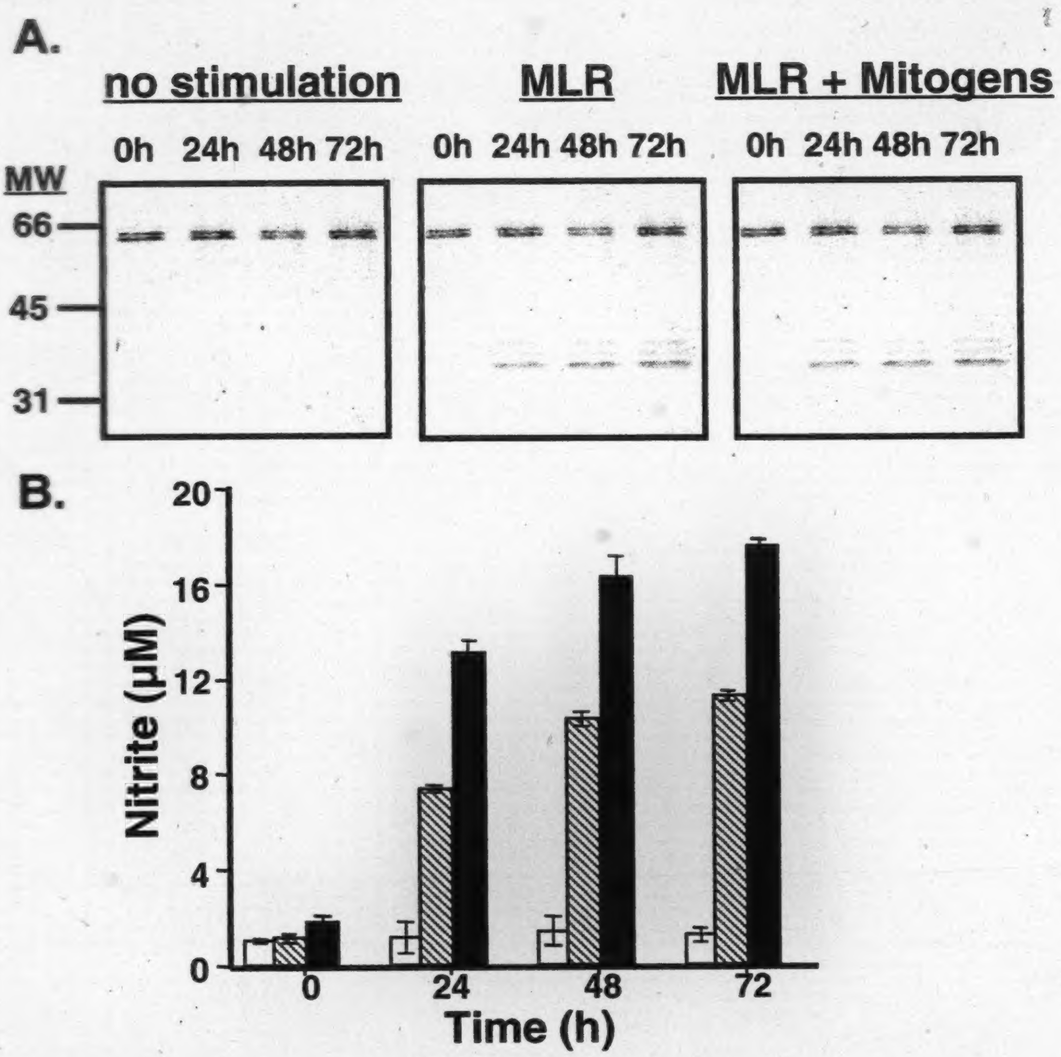

Figure 5.2. Time-course analysis of mitogen-stimulated goldfish kidney leukocyte supernatants by Western blot and the NO bioassay. Kidney leukocytes from an individual fish were seeded into a tissue culture flask ($4x10^6$/ml) in the absence of mitogens (no stimulation; white bars) or kidney leukocytes from 20 individual fish were pooled (MLR; thatched bars) and/or stimulated with mitogens (MLR + mitogens; black bars). Sub-samples (500 µl) were removed from each flask at 0, 24, 48, and 72 h following stimulation and analyzed by Western blot using the anti-NO-inducing factor antibody (8C2) (A). Sub-samples were also tested for NO-inducing activity by seeding goldfish macrophages into the wells of a 96-well (half-area) tissue culture plate ($5x10^4$/well). Cells were stimulated with the various sub-samples (1:4 dilution) in conjunction with LPS (1 µg/ml). Nitrite production was determined 72 h later using the Griess reaction and each bar represents the mean ± SEM of triplicate cultures and data are from a representative experiment of two independent experiments performed.

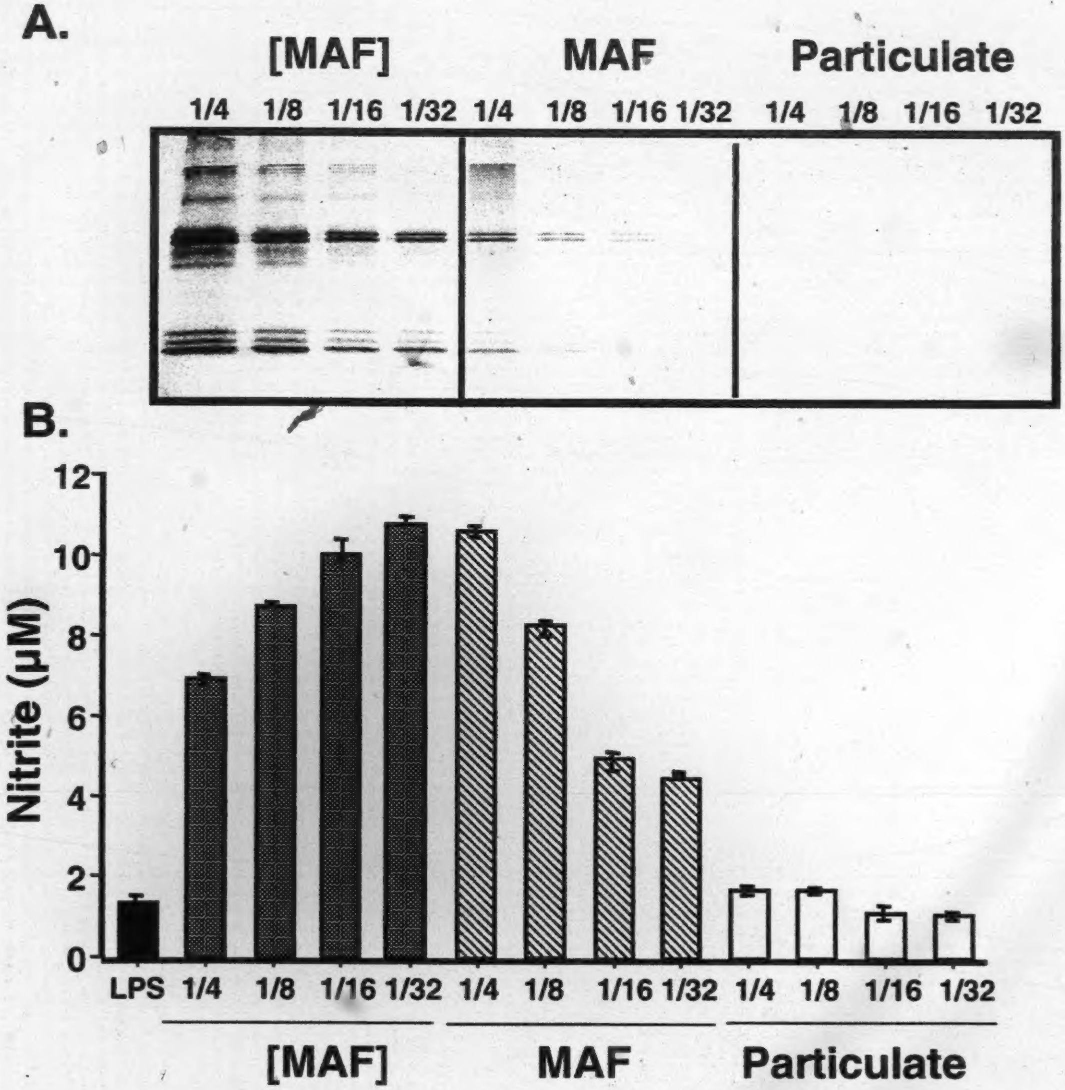

Figure 5.3. Concentration of mitogen-stimulated goldfish kidney leukocyte supernatants and examination by Western blot and the NO bioassay. Leukocyte supernatants were concentrated approximately 30-fold using PEG and the concentrated sample ([MAF]), unconcentrated sample (MAF), and re-dissolved particulate examined by Western blot using the anti-NO-inducing factor antibody (A). Samples were also tested for NO-inducing activity by incubating goldfish macrophages ($5x10^4$/well) in 96-well half-area tissue culture plates with various dilutions of the samples in conjunction with 1 μg/ml LPS (B). Nitrite production was determined 72 h later using Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and data are from a representative experiment of three independent experiments performed.

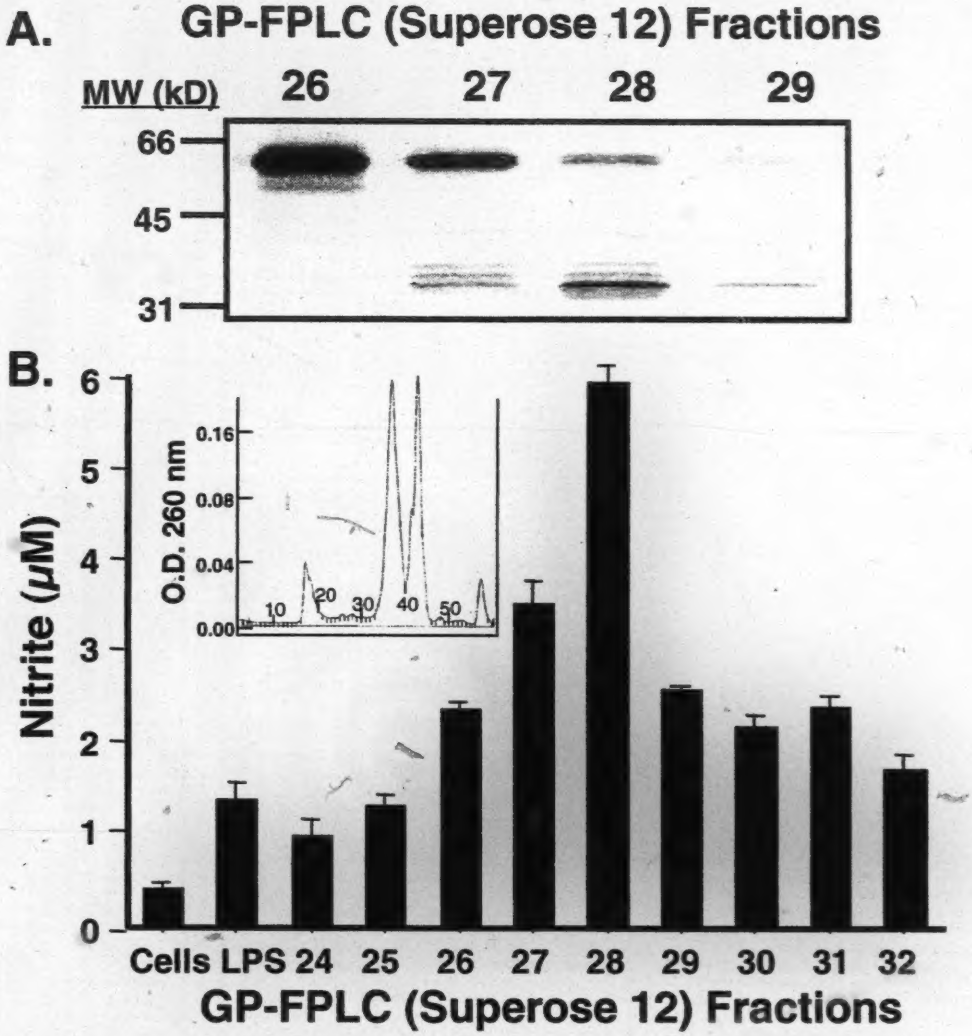

Figure 5.4. GP-FPLC separation of mitogen-stimulated goldfish kidney leukocyte supernatants. Leukocyte supernatants were separated using a Superose 12 size-exclusion column, and the individual fractions were analyzed by Western blot using the anti-NO-inducing factor (8C2) as the primary antibody (A). Nitric oxide-inducing activity of the individual fractions was determined using the NO bioassay (B). Day 8-10 goldfish macrophages were seeded into wells of a half area 96 well culture plate ($5x10^4$ cells/well) and incubated with individual fractions from GP-FPLC separated mitogen-stimulated leukocyte supernatants (1:4) and LPS (1 μg/ml). Nitrite production was determined 72 h later using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and is representative of three independent experiments that were performed. Inset: a typical Superose 12 chromatography trace of fractionated leukocyte supernatants.

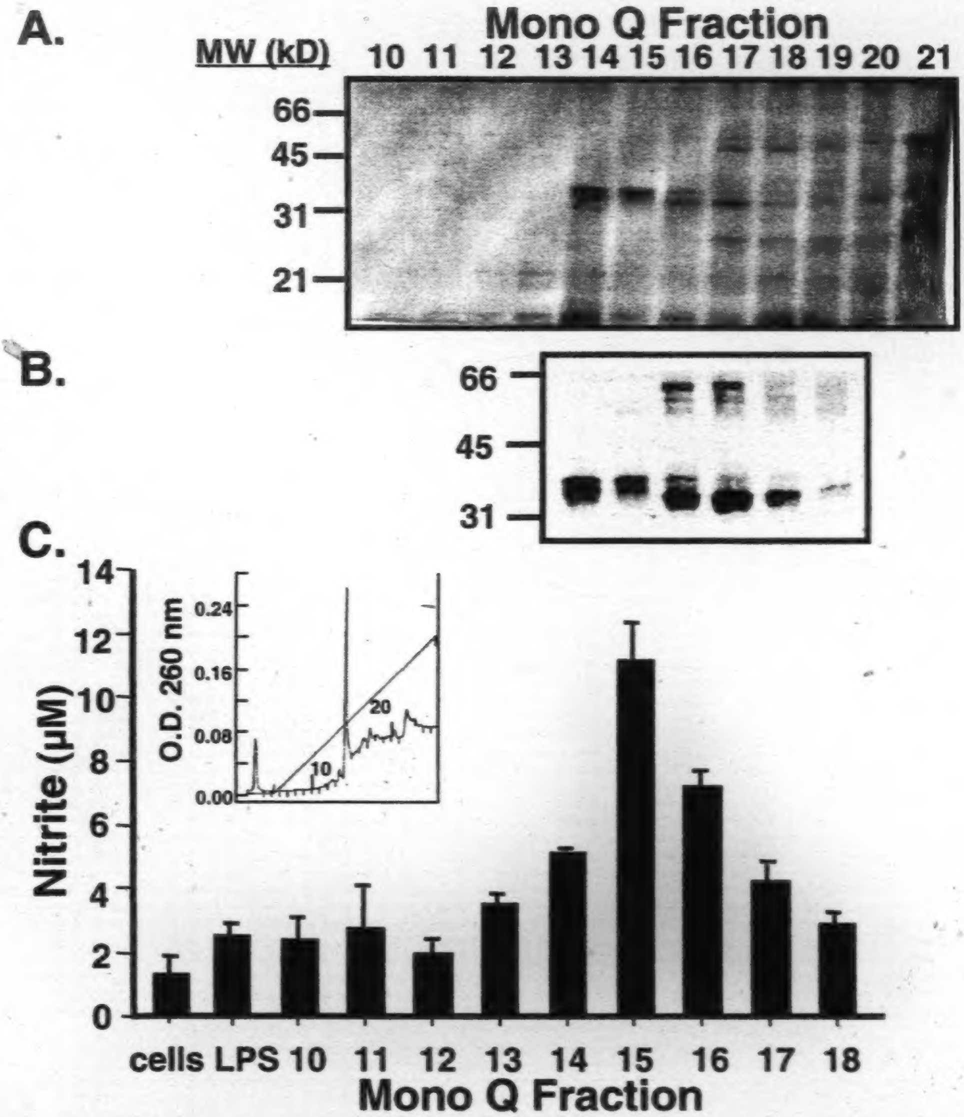

Figure 5.5. Ion-exchange chromatography of mitogen-stimulated goldfish kidney leukocyte supernatants. Supernatants were separated using a Mono-Q column and the individual fractions analyzed by non-reducing SDS-PAGE and the protein bands visualized by silver stain (A) or by Western blot using the anti-NO-inducing factor antibody (8C2; 1:400) (B). Nitric oxide-inducing activity of the individual fractions was determined using the NO bioassay (C). Day 8-10 goldfish macrophages were seeded into wells of a half area 96 well culture plate ($5x10^4$ cells/well) and incubated with individual fractions from Mono-Q separated mitogen-stimulated leukocyte supernatants (1:4) and LPS (1 µg/ml). Nitrite production was determined 72 h later using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and is representative of three independent experiments that were performed. Inset: a typical Mono-Q chromatography.

MRPAVRALLACAVLGLCLADPERTVRWCTISTHEANKCAS
FRENVLRILESGPFVSCVKK**TSHMDCIK**AISNNEADAVTLD
GGLVYEAGLKPNNLKPVVAEFHGTK**DNPQTHYYAVAVVK**
KDTDFKLNELRGKKSCHTGLGRSAGWNIPMAK**LYKELPD**
PQESIQRAAANFFSASCVPCADQSSFPKLCQLCAGKGTDK
CACSNHEPYFGYSGAFKCLMEGA**GDVAFVKHSTVFDNLP**
NPEDRKNYELLCGDNTRKSVDDYQECYLAMVPSHAVVA
RTVGGKEDVIWELLNHAQEHFGK**DKPDNFQLFQSPHGK**D
LLFK**DSADGFLK**IPSKMDFELYLGYEYVTALQNLRESKPPD
SSKDECMVKWCAIGHQERTKCDRWSGFSGGAIECETAEN
TEECIAKIMK**GEADAMSLDGGYLYIAGK**CGLVPVLAENYK
TEGESCKNTPEK**GYLAVAVVKTSDANINWNNLK**DKKSCH
TAVDR**TAGWNIPMGLLYSK**INNCK**FDEFFSAGCAPGSPR**
NSSLCALCIGSEKGTGKECVPNSNERYYGYTGAFR**CLVEK**
GDVAFVKDQTVIQNTDGNNNEAWAKNLKKENFEVLCKDG
TRKPVTDAENCHLAR**GPNHAVVSR**KDKATCVEKILNK**QQ**
DDFGKSVTDCTSNFCLFQSNSKDLLFRDDTKCLASIAK**KT**
YDSYLGDDYVRAMTNLRQCSTSKLLEACTFHKP

Figure 5.6. Identification of peptide fragments from mass-spectrometry analysis of FPLC purified goldfish macrophage NO-inducing factor. Protein bands with estimated MW of 33, 35, and 37 kD were analyzed by mass spectrometry (Harvard Microchemistry). All three proteins were identified as serotransferrin precursor (Siderophilin) GenBank Accession# U02564. Shown is the predicted amino acid sequence of GenBank #U02564. Bold letters denote the fragments identified by mass spectrometry. Underlined is the bridge peptide that separated the N-lobe from the C-lobe of transferrin.

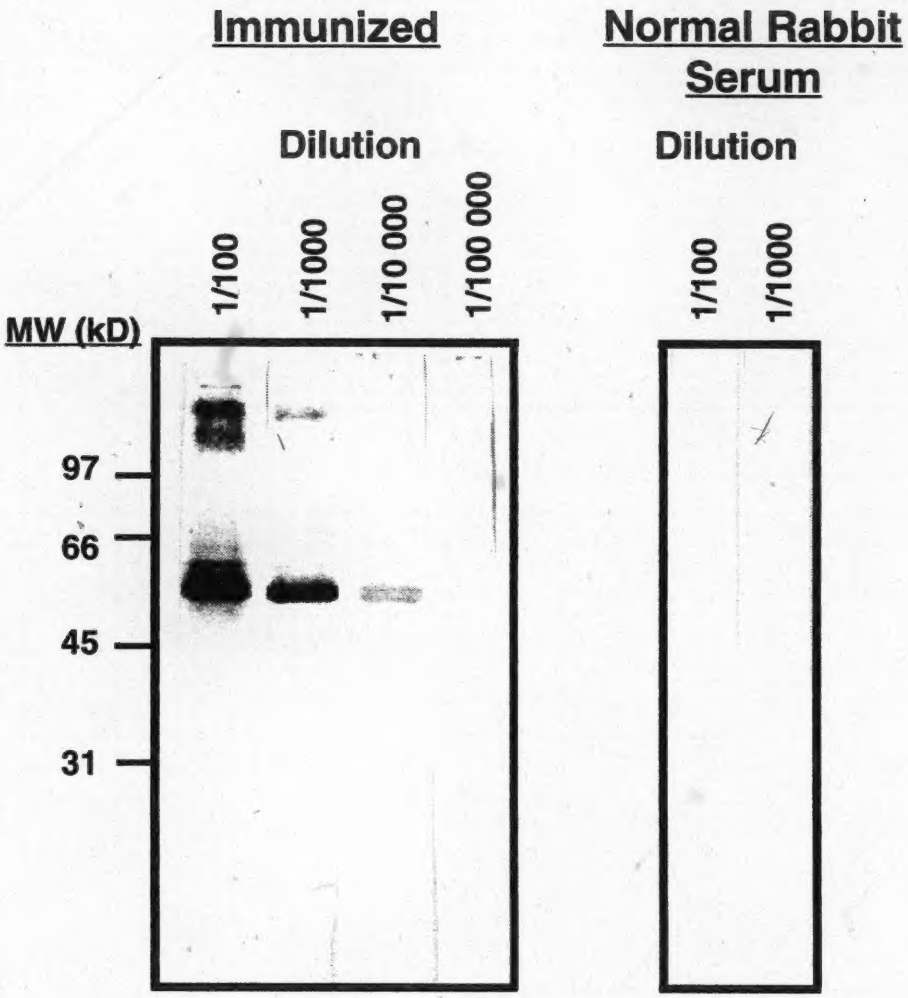

Figure 5.7. Immunoreactivty of anti-NO-inducing factor antibody. Bovine holo-transferrin (1 μg) was separated by non-reducing SDS-PAGE and transferred to a 0.2 μm nitrocellulose membrane. Individual lanes containing 1 μg of transferrin were cut into strips and incubated with anti-NO-inducing factor antibody (8C2) at dilutions ranging from 1:100 to 1:100 000. Normal rabbit serum from a non-immunized rabbit was included as a control.

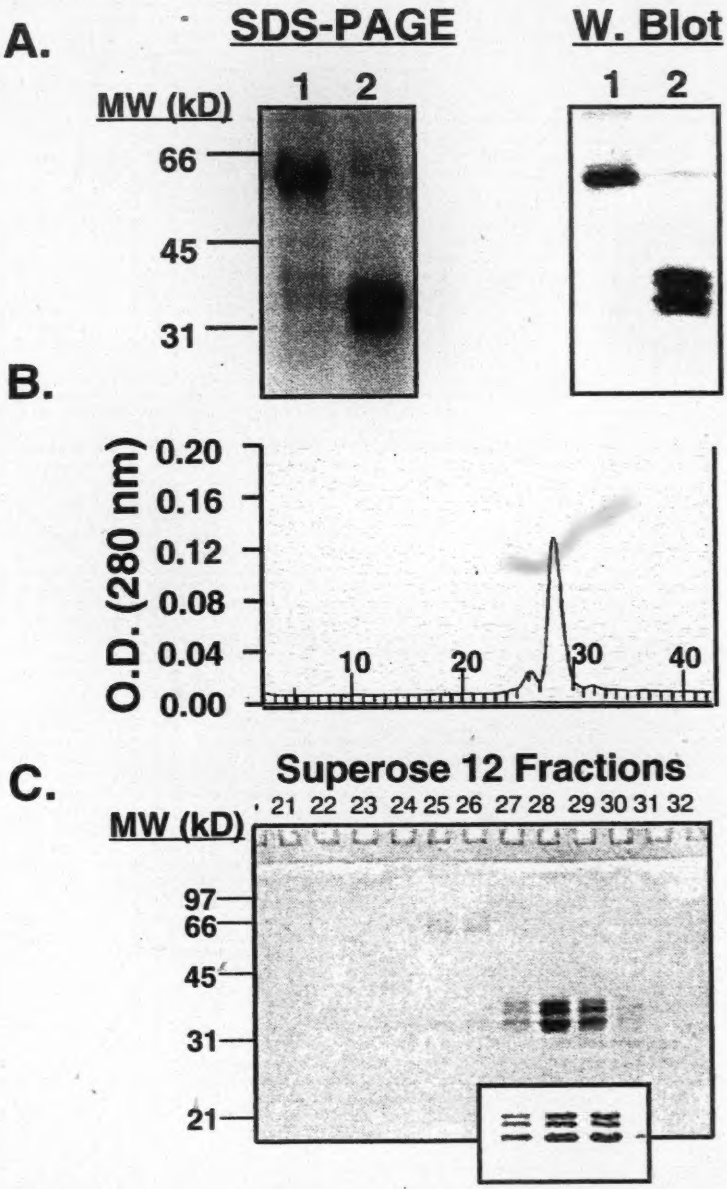

Figure 5.8. Protease-digestion and GP-FPLC separation of commercially purchased transferrin. Bovine transferrin (Sigma) was protease-digested for 20 h at room temperature. Undigested (Lane 1) and digested (Lane 2) transferrin samples were separated by non-reducing SDS-PAGE and analyzed by Western blot using the anti-NO-inducing factor (8C2) antibody (A). Transferrin fragments were then separated by GP-FPLC (Superose 12) (B) and protein bands visualized after non-reducing SDS-PAGE by Coomassie staining and Western blot (inset) using the anti-NO-inducing factor antibody (8C2) (C).

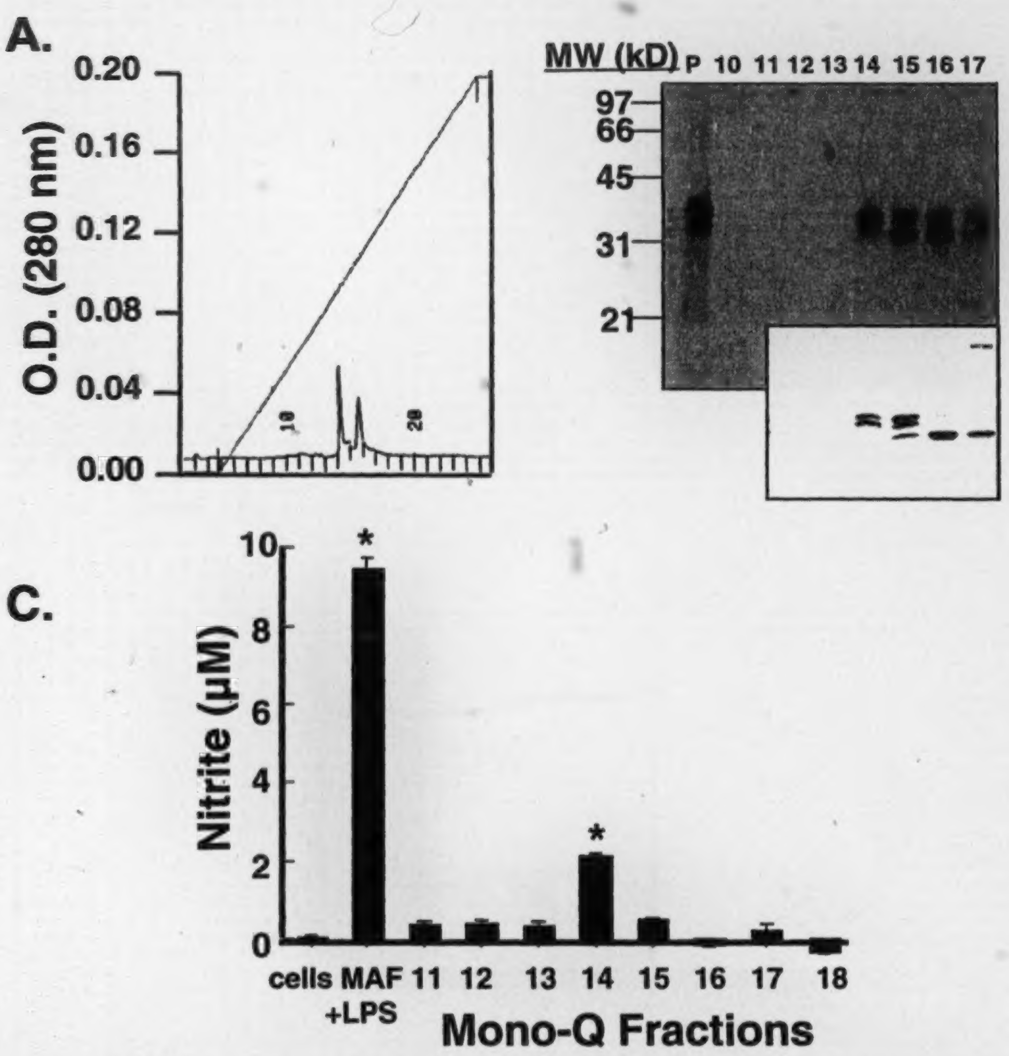

Figure 5.9. Ion-exchange chromatography of protease-digested transferrin and NO-inducing activity of the cleaved fragments. GP-FPLC fractions that contained the majority of the digested transferrin (i.e. fractions #27-30; Fig.5.8) were pooled and further separated by ion-exchange chromatography (Mono-Q) (A). Individual Mono-Q fractions were separated by non-reducing SDS-PAGE and the protein bands visualized by silver staining and Western blot (insert) using the anti-NO-inducing factor antibody (A). Lane (P) represents the pooled sample, which was injected onto the Mono-Q column. Individual fractions of Mono-Q separated protease-digested bovine transferrin were also tested for NO inducing activity (B). Day 8-10 goldfish macrophages were seeded into wells of a half area 96 well culture plate ($5x10^4$ cells/well) and incubated with individual Mono-Q fractions (1:4) and LPS (1 µg/ml). Nitrite production was determined 72 h later using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and is representative of three independent experiments that were performed. *P<0.0001 vs. cells alone group.

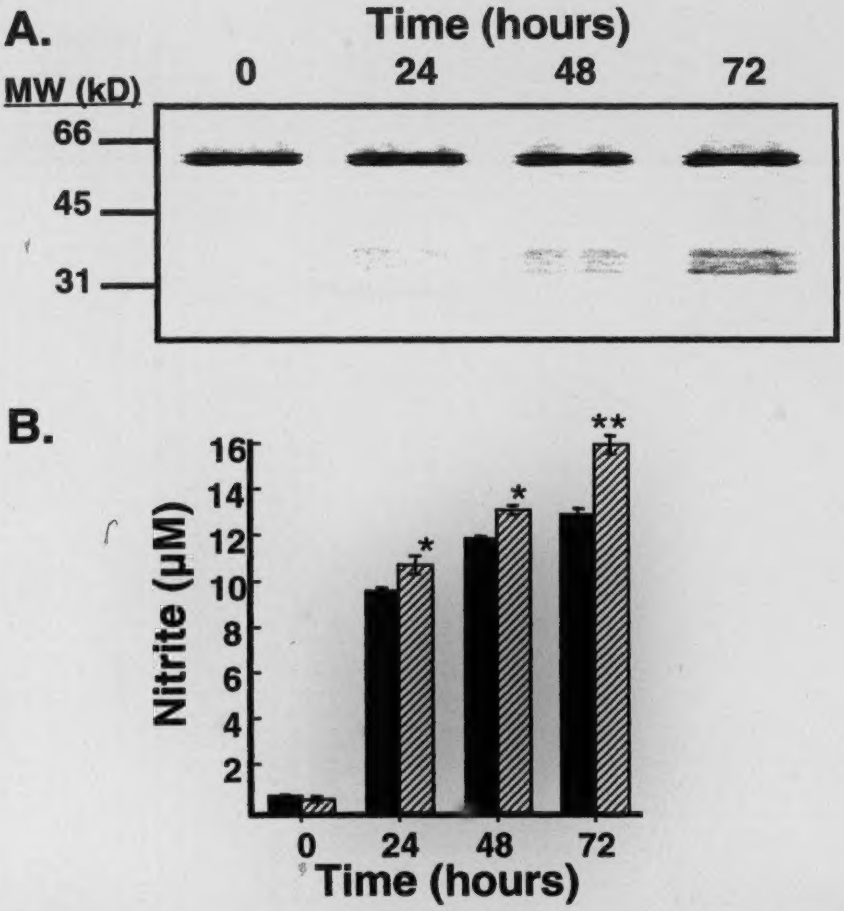

Figure 5.10. Enhanced NO-inducing activity of mitogen-stimulated goldfish kidney leukocyte supernatants by the addition of transferrin. Goldfish kidney leukocytes were isolated and stimulated with mitogens after an overnight (~18 h) incubation with 5% carp serum only. After 6 h the mitogens and serum were removed, and the cells incubated in serum-free medium alone (black bars) or serum-free medium supplemented with 25 µg/ml of bovine transferring (hatched bars). Sub-samples were removed after 0, 24, 48, and 72 h following mitogen-stimulation and analyzed by Western blot using the anti-NO-inducing factor antibody (8C2) (A) Sub-samples were also tested for NO-inducing activity (B). Nitric oxide-inducing activity of the sub-samples was determined by seeding goldfish macrophages (8-12 days old) into wells of half area 96 well culture plates at a density of 5x10^{4} cells/well. Cells were stimulated with the various sub-samples (1:4 dilution) and LPS (1 µg/ml). Nitrite production was determined 72 h later using the Griess reaction and each bar represents the mean ± SEM of triplicate cultures and results are from a representative experiment of three that were performed. [Note: * represents $P<0.05$, and ** represents $P<0.0001$].

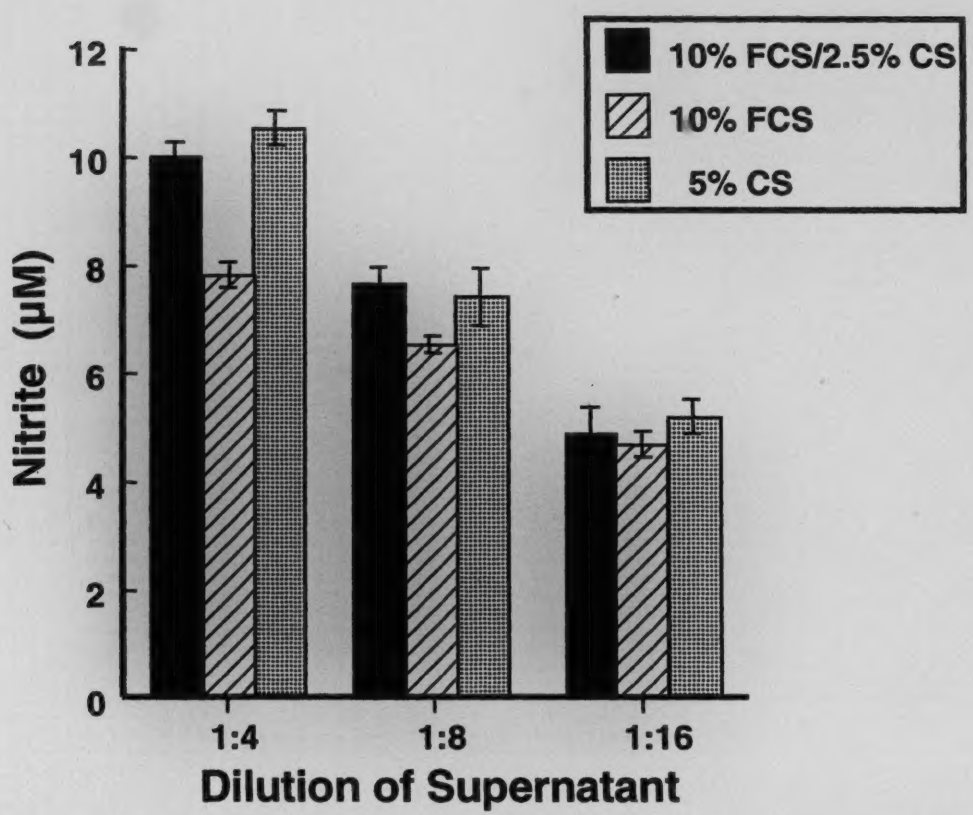

Figure 5.11. NO-inducing activity of mitogen-stimulated goldfish kidney leukocyte supernatants generated in the presence of bovine and/or carp serum. Mitogen-stimulated goldfish kidney leukocyte supernatants were prepared and prior to mitogen-stimulation, the leukocytes were incubated overnight in the presence of 10% FCS and 2.5% carp serum, 10% FCS alone, or with 5% carp serum alone. After 6 h, mitogens were removed and cells incubated in serum-free medium for a further 72 h. After 72 h, the supernatants were collected and tested for NO-inducing activity by seeding goldfish macrophages (8-12 days old) into half area 96 well culture plates at a density of 5x10^4 cells/well and stimulating with a 1:4 dilution of the various supernatants in conjunction with 1 µg/ml LPS. Nitrite production was determined 72 h later using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and is representative of two independent experiments that were performed.

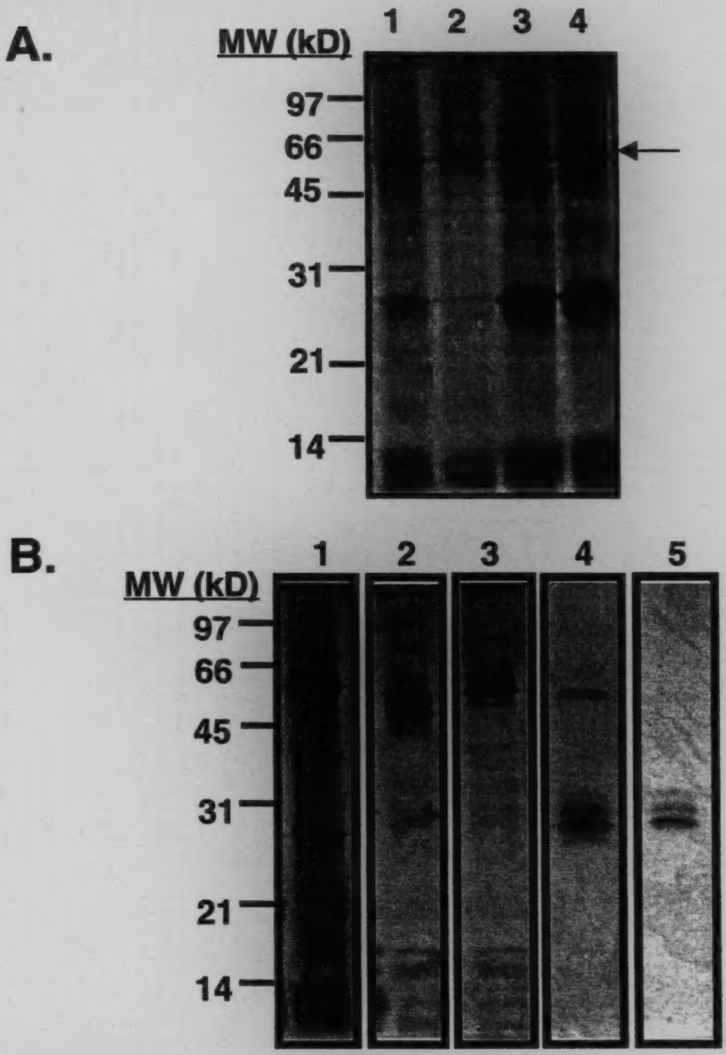

Figure 5.12. Purification of carp serum transferrin cleavage products. Carp serum was dialyzed overnight vs. different DEAE-Affi-Gel Blue binding buffers to determine the optimal conditions for purification of carp transferrin and the samples were analyzed by non-reducing SDS-PAGE and silver staining (A). Lane 1, 20 mM Tris-HCL, 25 mM NaCl pH 9.3; Lane 2, 20 mM Tris-HCL, 25 mM NaCl pH 8.0; Lane 3, 20 mM K_2HPO_4 pH 7.0; Lane 4, K_2HPO_4 pH 8.0. Carp serum transferrin (indicated by arrow) was then further purified using a combination of Con A Sepharose chromatography and ion-exchange chromatography (B) Lane 1; sample eluted from DEAE column using 20 mM Tris-HCL, 25 mM NaCl pH 9.3; Lane 2, Con A bound fraction; Lane 3, Con A unbound fraction; Lane 4, protease digestion of Con A unbound fraction; Lane 5, Mono-Q fraction#13 of separated protease-digested Con A unbound fraction.

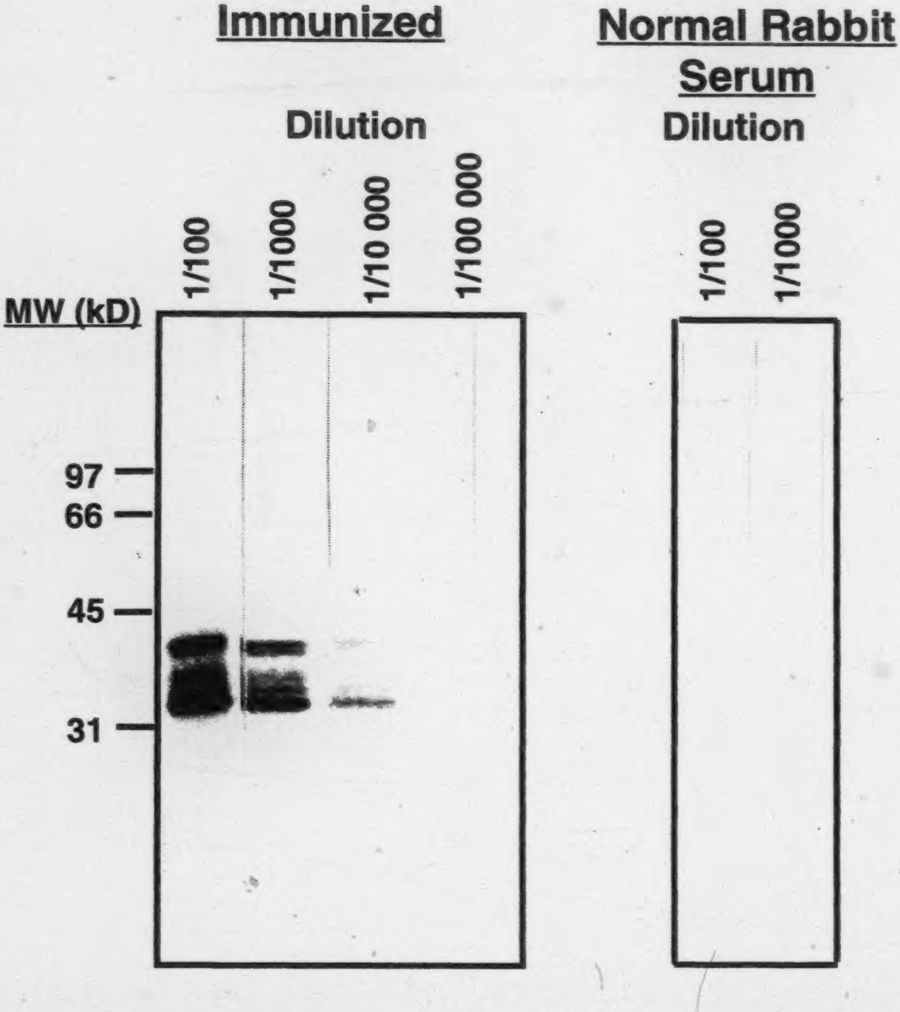

Figure 5.13. Immunoreactivty of anti-carp transferrin polyclonal antibody. Con A purified carp serum transferrin samples (1 μg) were separated by non-reducing SDS-PAGE and transferred to 0.2 μm nitrocellulose. Individual lanes containing 1 μg of carp transferrin were cut into strips and incubated with anti-carp transferrin antibody (9AG7) at dilutions ranging from 1:100 to 1:100 000. Normal rabbit serum from a non-immunized rabbit was included as a control.

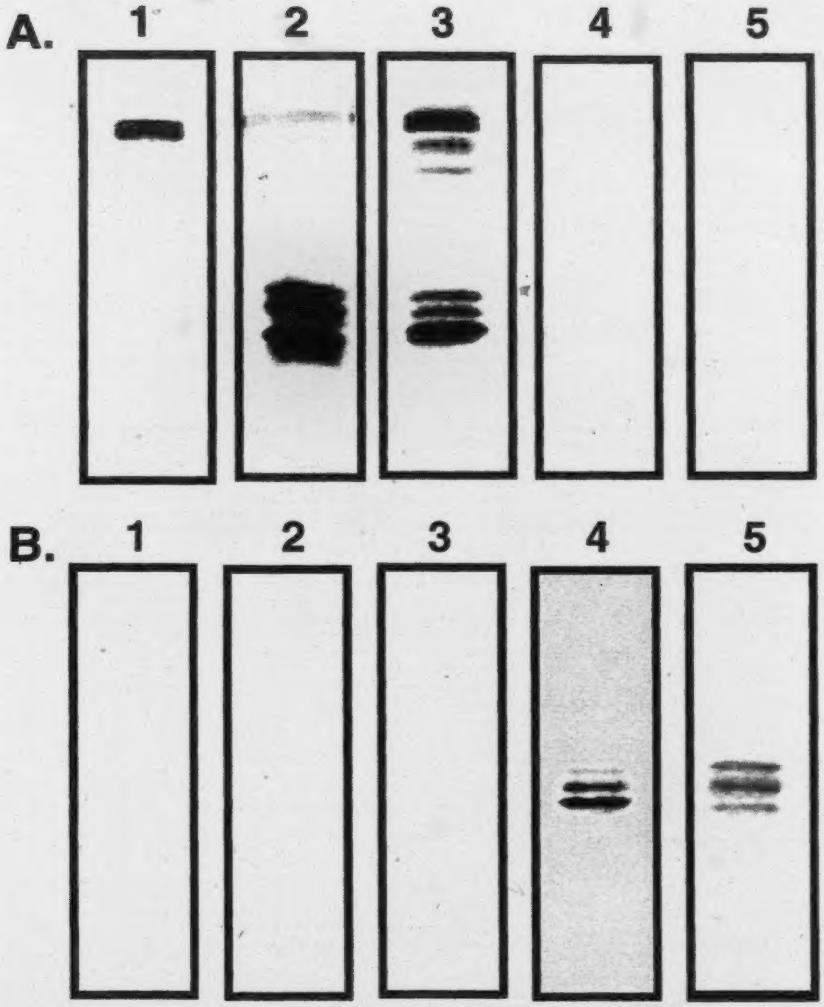

Figure 5.14. Western blot analysis using anti-NO-inducing factor (8C2) and anti-carp serum transferrin (9AG7) polyclonal antibodies. Samples were separated by non-reducing SDS-PAGE and transferred to 0.2 µm nitrocellulose membranes. Blot (A) was reacted with 8C2 and blot (B) reacted with 9AG7. The samples loaded into each lane are as follows: Lane 1: commercially purchased (Sigma) bovine transferrin; Lane 2: protease-digested bovine transferrin (Sigma); Lane 3: crude mitogen-stimulated leukocyte supernatant prepared in the presence of 10% FCS only; Lane 4: Ion-exchange (Mono-Q) purified carp serum transferrin cleavage products; Lane 5: mitogen-stimulated leukocyte supernatant prepared in the presence of 5% carp serum only (Note: this lane represents a concentrated sample after GP-FPLC which contained maximal NO-inducing activity; i.e. Superose 12 fraction #29).

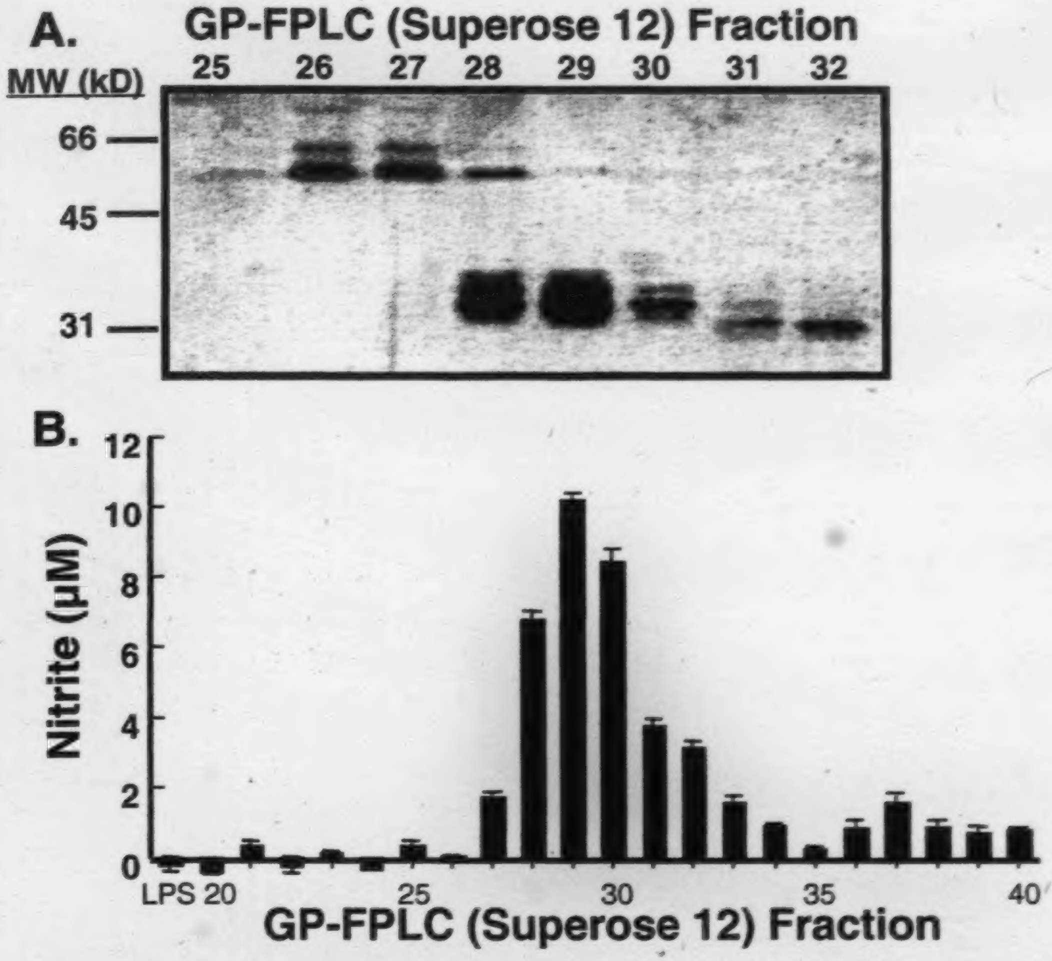

Figure 5.15. Detection of carp serum transferrin cleavage products in the GP-FPLC fractions exhibiting maximal NO-inducing factor activity. Con A purified carp serum transferrin was protease digested and then separated by using a Superose 12 column and individual fractions analyzed by non-reducing SDS-PAGE and proteins visualized by silver staining (A). GP-FPLC separation and NO-inducing activity of mitogen-stimulated goldfish kidney leukocyte supernatants prepared following overnight incubation with 5% carp serum only (B). Leukocyte supernatants were separated using a Superose 12 column, and NO-inducing activity of individual fractions were determined using the NO bioassay. Day 8-10 goldfish macrophages were seeded into wells of a half-area 96 well culture plate ($5x10^4$ cells/well) and stimulated with the individual fractions from GP-FPLC separated mitogen-stimulated leukocyte supernatants (1:4) and LPS (1 µg/ml). Nitrite production was determined 72 h later using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and is representative of three independent experiments that were performed.

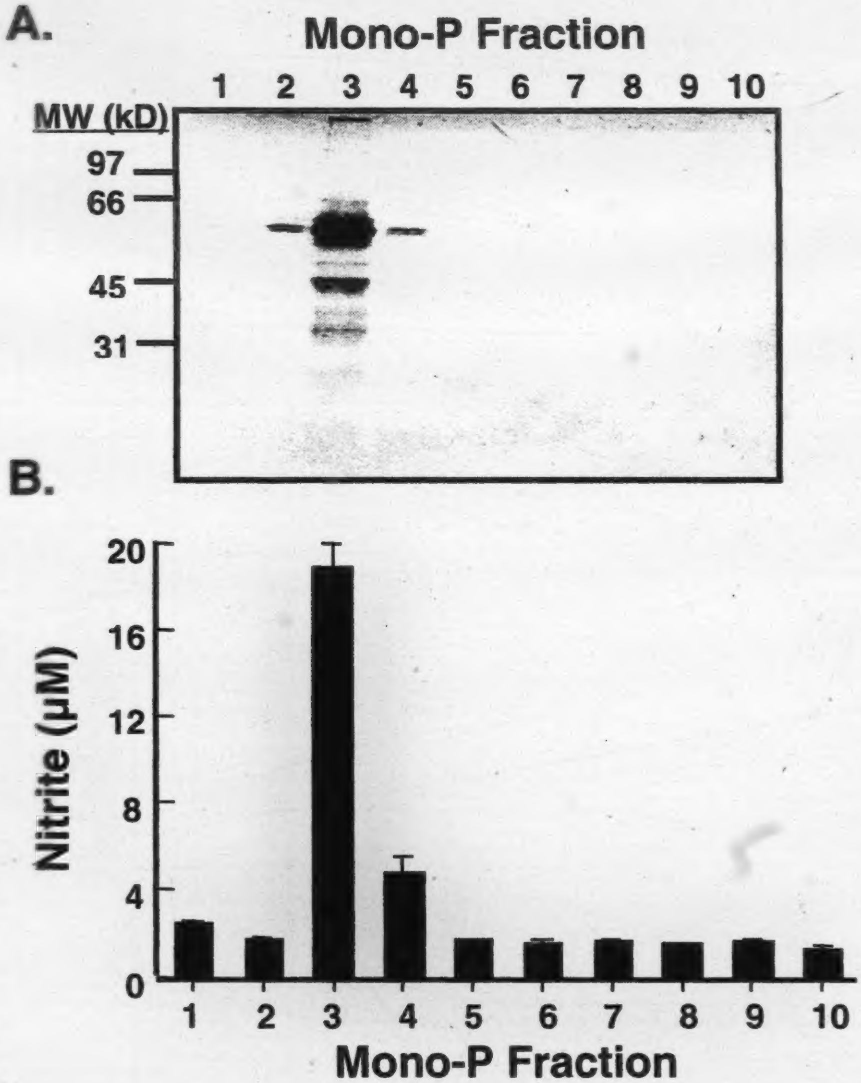

Figure 5.16. C-FPLC separation and Western blot of mitogen-stimulated goldfish kidney leukocyte supernatants prepared in the presence of goldfish serum. Leukocyte supernatants prepared in the presence of 5% goldfish serum were separated using a Mono-P column with a pH gradient of 7.0-5.0. Individual fractions were then separated by SDS-PAGE and the bands visualized following incubation with anti-carp transferrin (9AG7) antibody (A). The NO-inducing activities of individual Mono-P fractionated leukocyte supernatants was determined by seeding (5x10^4 cells/well) 8-12 day old goldfish macrophages into wells of a half-area 96 well culture plate and stimulating with the individual fractions Mono-P fractions (1:4) and LPS (1 µg/ml). Nitrite production was determined 72 h later using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and is representative of three independent experiments that were performed.

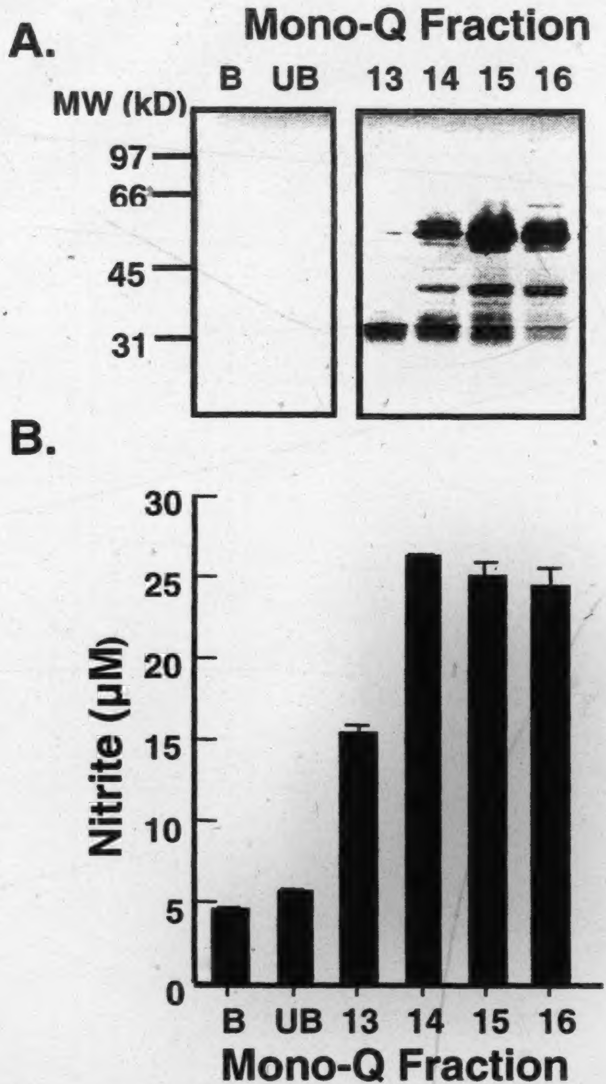

Figure 5.17. Ion-exchange (Mono-Q) separation and Western blot analysis of leukocyte supernatants prepared in the presence of 5% goldfish serum. Individual Mono-Q fractions were separated by SDS-PAGE and the bands visualized following incubation with anti-carp transferrin (9AG7) antibody (A). The NO-inducing activities of individual Mono-Q fractionated leukocyte supernatants was determined by seeding ($5x10^4$ cells/well) 8-12 day old goldfish macrophages into wells of a half-area 96 well culture plate and stimulating with Mono-Q running buffer (B; 1:4), a flow-through (i.e. unbound) column fraction (UB; Mono-Q fraction #5 (1:4)), or individual Mono-Q fractions (1:4) and LPS (1 µg/ml); (B) . Nitrite production was determined 72 h later using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and is representative of three independent experiments that were performed.

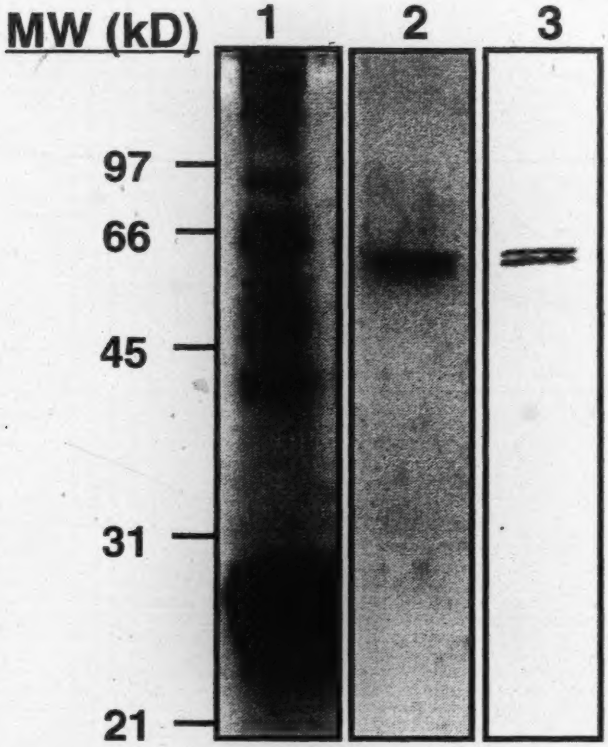

Figure 5.18. Immunopurification of goldfish transferrin using the polyclonal anti-carp serum transferrin polyclonal antibody. Pure polyclonal IgG was isolated from immunized rabbit serum as described in the materials and methods section and ligated 1 ml HiTrap NHS-activated HP column. DEAE partially-purified goldfish serum was than applied to the immunoaffinity column and bound proteins eluted with 0.5 M acetic acid, pH 3.4. Samples were then analyzed by non-reducing SDS-PAGE and Western blot. Lane 1, immunoaffintiy unbound fraction; Lane 2, immunoaffinity bound fraction; Lane 3, Western blot of Lane 2 using the anti-carp serum transferrin polyclonal IgG.

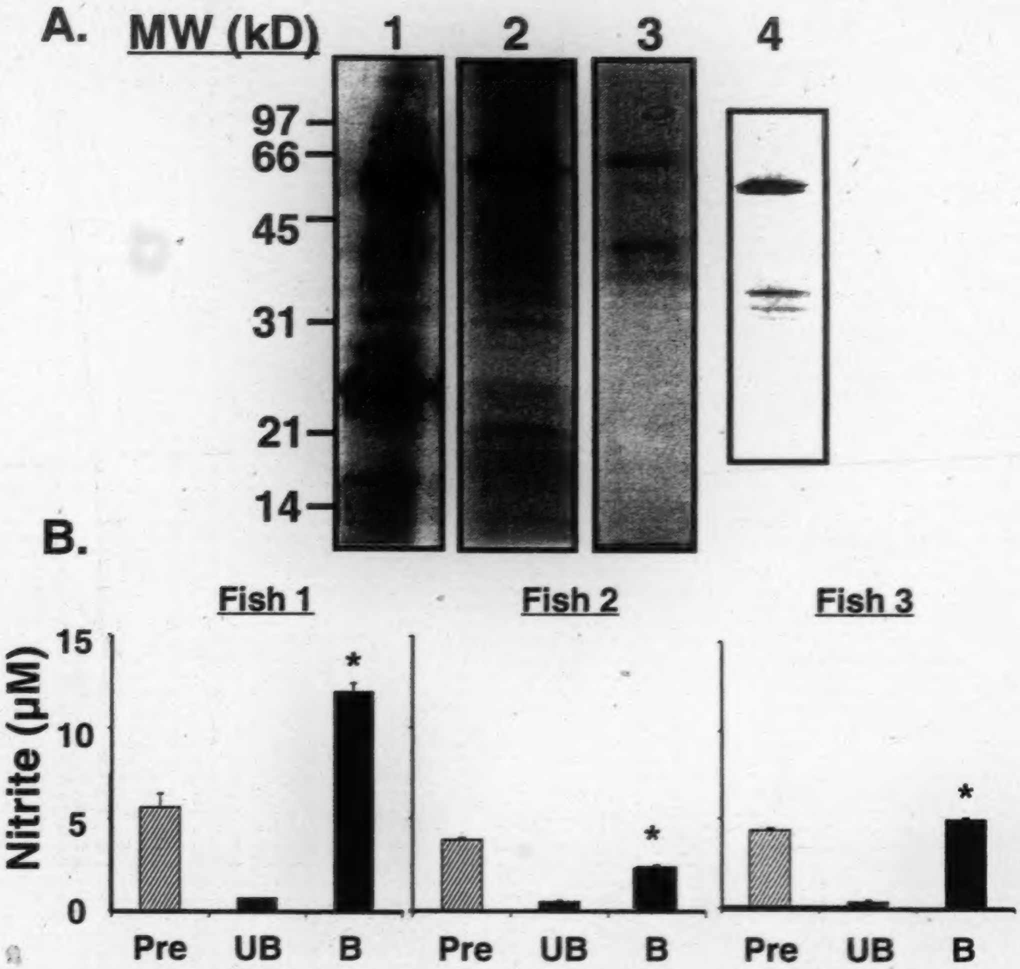

Figure 5.19. Immunopurification of goldfish transferrin from mitogen-stimulated goldfish kidney leukocyte supernatants. Polyclonal anti-carp transferrin IgG was conjugated to a 1 ml HiTrap NHS-activated HP column Partially-purified leukocyte supernatants (i.e. pooled MQ fractions #13-16; see Fig 5.17) were applied to the anti-carp transferrin immunoaffinity column and bound proteins eluted with 0.5 M acetic acid pH 3.4. Pre-affinity (Pre), unbound (UB) and bound (B) proteins (Lanes 1-3, respectively) were separated by SDS-PAGE and visualized by silver staining. Lane 4 shows Western blot analysis of the bound fraction using the anti-carp serum transferrin polyclonal antibody (A). Immunoaffinity fractions were also tested for NO-inducing activity (B). Each bar represents the mean ± SEM of triplicate cultures and the results from three individual fish is shown. Results are from a representative experiment of two independent experiments that were performed. * represents a significant difference ($P<0.05$) when comparing the nitrate induced by unbound and bound immunoaffinity fractions.

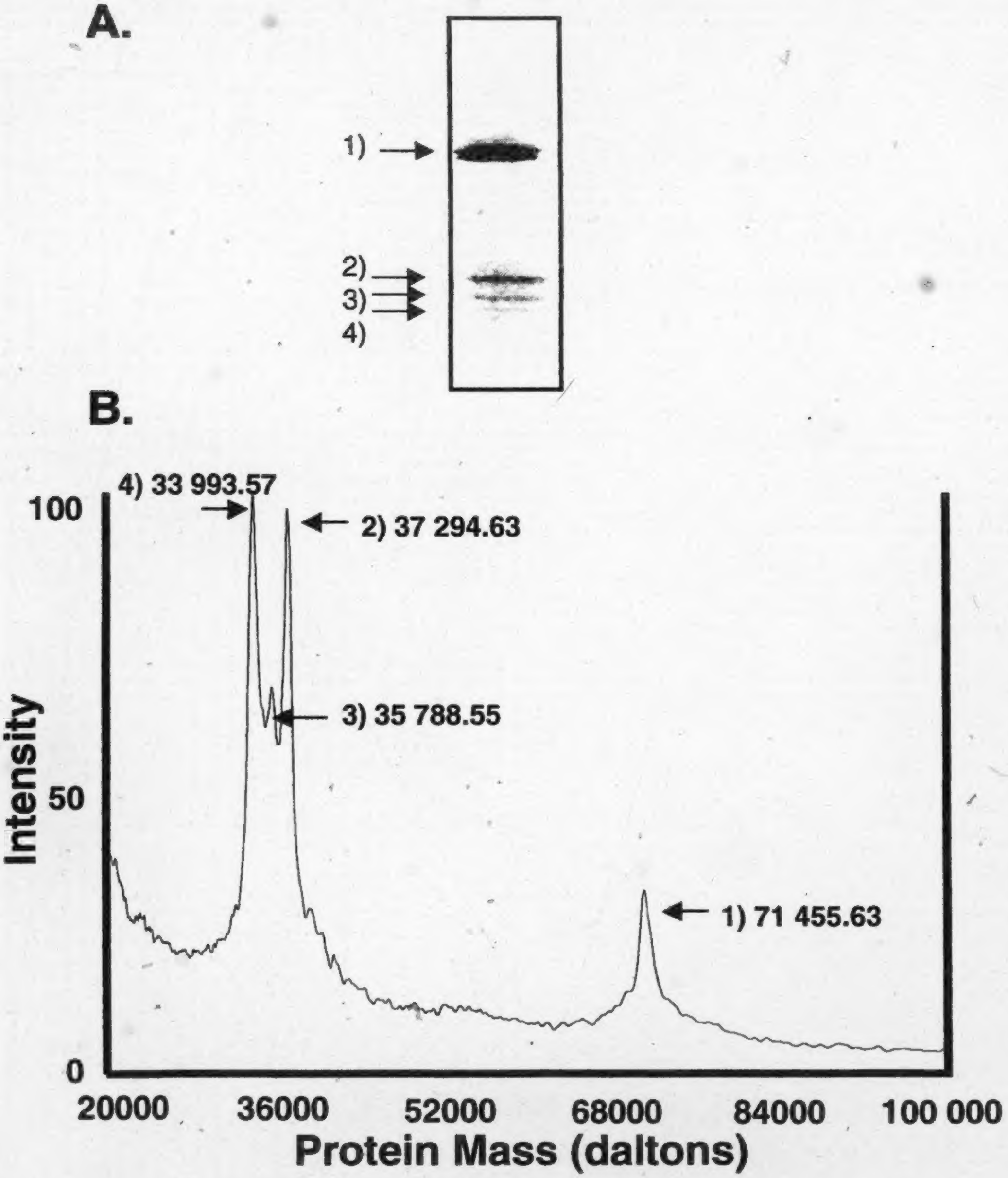

Figure 5.20. Mass spectrometry (MALDI-TOF) analysis of immunopurified goldfish transferrin. Goldfish transferrin was immunopurified from mitogen-stimulated goldfish kidney leukocyte supernatants as described in the materials and method section and sent to the University of Victoria Genome BC Proteomics Centre (www.proteincentre.com) for analysis. Immunopurified samples consisted of four distinct protein bands detected by Western blot (A), which correlated with proteins of 71 455.63, 37 294.63, 35 788.55, and 33 993.57 daltons as determined by MALDI-TOF analysis (B).

CHAPTER 6

BIOLOGICAL CHARACTERIZATION OF RECOMBINANT GOLDFISH TRANSFERRIN AND ITS ROLE IN MACROPHAGE ACTIVATION[5]

6.1 Introduction

The biological and functional characterization of a leukocyte-derived NO-inducing factor identified that transferrin cleavage products play an important role in the activation of goldfish macrophages (Chapter 5). Induction of an innate immune response by transferrin cleavage products is novel, however the recognition of fragments from ubiquitously distributed endogenous molecules appears to be a highly conserved mechanism of immune activation. Similar processes have been observed in plants and invertebrates and this may also be an important process in vertebrates such as fish and mammals (described below).

Injury and/or infection result in the release of a variety of enzymes, which leads to the subsequent degradation of endogenous molecules at the tissue site. For example, inflammation causes the shedding of heparan sulfate from mammalian cells [637-641]. The activation of the complement cascade and neutrophils, as well as the low pH of damaged tissue, generated soluble heparan sulfate proteoglycan and induced its subsequent fragmentation [639-640, 642-646]. One of the key enzymes responsible for the fragmentation of ubiquitously distributed endogenous molecules like heparan sulphate is the serine protease, elastase [644, 646]. Soluble heparan sulfate is not present in significant quantities in healthy tissues, thus it has recently been proposed that receptor-mediated monitoring of tissue health is accomplished by the detection of this endogenous molecule in damaged tissues by immune cells [156].

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[5] A version of this chapter has been published: Stafford and Belosevic, 2003. *Dev. Comp. Immunol.*, 27: 539-554.

In plants and invertebrates, the recognition of fragments derived from ubiquitously distributed endogenous proteins generated during tissue injury and/or infection is performed by sentinel immune receptors [647-648]. Interaction of degraded endogenous molecules with these sentinel receptors induces innate immune responses [649]. The sentinel receptors that are responsible for recognizing fragments of endogenous molecules belong to a family of highly conserved proteins such as *Drosophilia* Toll, which are homologous to plant resistance proteins (R proteins) [647, 650-651]. The recently discovered vertebrate Toll-like receptors (TLRs) share significant homologies with these plant and invertebrate innate immune receptors. Vertebrate TLRs recognize products released from bacterial pathogens (i.e. endotoxin) [650-652], as well as endogenously-derived proteins including fibrinogen, fibronectin and heat-shock proteins [10-11, 153]. For example, dendritic TLR-4 was identified as the receptor for fragments of heparan sulfate generated during tissue injury and a model of receptor-mediated monitoring of tissue homeostasis was proposed that requires highly conserved innate immune receptors that monitor tissues for signs of infection or injury [156]. These authors also suggested that sentinel receptors in plants and invertebrates, as well as highly evolved innate immune receptors in vertebrates, are capable of recognizing general signals of tissue disease manifested by fragmentation of endogenous molecules. However, to date, only polysaccharide fragments of heparan sulfate proteoglycan have been identified as a ubiquitously distributed endogenous molecule capable of stimulating vertebrate immune responses by interacting with innate immune receptors such as TLR-4 [151, 156]. Thus it is intriguing to speculate that cleavage products derived from transferrin at sites of tissue injury or infection may also function through a similarly conserved mechanism in fish.

In this chapter, I describe the results of experiments designed to identify a potential cleavage-site required for the generation of active transferrin fragments, and demonstrate that this predicted cleavage site is conserved between different species. I also report on the source(s) of the enzyme(s) responsible for this activity, cloning of the goldfish transferrin gene, expression of the recombinant proteins (i.e. full-length goldfish transferrin and N- and C-lobe fragments), and the functional characterization of these recombinants. In addition, I describe how these proteins were expressed using both prokaryotic and eukaryotic expression systems. The potential conservation of transferrin-mediated macrophage activation was addressed by testing the NO-inducing activities of recombinant goldfish transferrin on macrophages of goldfish and mice.

6.2 Results

6.2.1 Detection of tranferrin cleaving activity in mitogen-stimulated goldfish kidney leukocyte supernatants and lysed goldfish leukocytes

Examination of mitogen-stimulated goldfish kidney leukocytes by microscopy revealed that there was a significant amount of cellular lysis and death up to 72 h post-mitogen stimulation (Fig. 6.1A); the time-point when these supernatants were collected for purification of the NO-inducing factor. Immediately following isolation, kidney leukocytes were non-adherent and viable with >95% of cells excluding trypan blue (data not shown). Leukocytes were then stimulated with mitogens for 6 h, and washed 3 times with 1 X HBSS. Following removal of the mitogens (i.e. 0 h post-stimulation; Fig. 6.1A) a significant increase in cellular adhesion and clumping representative of an 'activated' cell culture was observed. Cells were examined microscopically at 24, 48, and 72 h post-stimulation to demonstrate the significant increase in cellular lysis and debris present in stimulated leukocyte cultures. Products released from lysed/necrotic cells have been shown to exhibit pro-inflammatory properties [653]. Therefore, I decided to test whether supernatants from cultures containing necrotic/damaged cells could induce the cleavage of transferrin into active fragments. As shown in Fig. 6.1B, supernatants from mitogen-stimulated leukocytes contained transferrin-cleaving activity that was observed as early as 15 min. Maximal cleaving activity was observed after longer incubation times (i.e. 4 h incubation). In addition, freeze-thaw lysates prepared from goldfish kidney granulocytes, cultured goldfish macrophages and freshly isolated goldfish kidney leukocytes all contained significant transferrin cleaving activity (Fig. 6.2).

6.2.2 Elastase generated transferrin fragments that induced NO production in goldfish macrophages

Incubation of elastase with goldfish serum or commercially purchased transferrin, resulted in the generation of transferrin cleavage products (Fig. 6.3). For example, co-incubation of goldfish serum with elastase resulted in the generation of a wide-range of smaller proteins when compared to the untreated serum following analysis by SDS-PAGE and coomassie blue staining (Fig. 6.3A). Analysis of elastase-treated goldfish serum by Western blot using the anti-carp serum transferrin polyclonal antibody (9AG7), demonstrated the presence of several smaller immunoreactive bands (i.e. transferrin fragments) that were not observed in the untreated serum (Fig. 6.3B). Elastase also cleaved commercially available transferrin into smaller fragments as determined by Western blot (Fig. 6.3C). These results suggested that elastase was one of the potential enzymes capable of cleaving native transferrin into immunostimulatory fragments. Furthermore, as shown in Fig. 6.3D, when macrophages were cultured in medium containing serum and then treated with elastase they produced NO. This effect was dose-dependent, and significant production of NO was only observed by goldfish macrophages incubated with 10 and 1 μg of enzyme. Therefore, co-incubation of goldfish macrophages with elastase in the presence of serum, resulted in the generation of transferrin cleavage products and the subsequent induction of NO. These results suggested that leukocyte-derived elastase may be one of the enzymes present in mitogen-stimulated goldfish kidney leukocyte supernatants that is capable of cleaving transferrin into its NO-inducing fragments.

6.2.3 Goldfish transferrin mRNA expression

RT-PCR and transferrin-specific primers were used to detect the expression of transferrin mRNA by goldfish liver and activated goldfish macrophages (Fig. 6.4). As expected, constitutive expression of transferrin was observed in goldfish liver and was detected using two different primer pairs that amplified a 620 bp and 1430 bp product (Fig. 6.4A). Interestingly, activated goldfish macrophages also expressed transferrin mRNA as early as 1 h post-activation, which reached maximal levels by 4-

6 h and declined at 12 and 24 h (Fig. 6.4B). Activation of goldfish macrophages was confirmed by monitoring the expression of the iNOS gene. Examination of kidney leukocytes at 0, 3, 6, and 24 h post-mitogen stimulation failed to demonstrate an increase in transferrin gene expression (Fig. 6.4C). A positive control (i.e. expression by liver tissue) was included to confirm detection of transferrin expression and a β-actin control included to confirm there was an intact cDNA template (Fig. 6.4C).

6.2.4 Prediction of goldfish transferrin cleavage site

The N- and C-terminal lobes of the native transferrin molecule are separated by an interdomain bridge peptide region that varies in length between different transferrin species [486-487]. The predicted amino acid sequence of goldfish transferrin is shown (Fig. 6.5A; GenBank Accession# AAK92216) and the putative bridge peptide is outlined. According to the sizes of the transferrin fragments following mass spectrometry analysis (Chapter 5), I hypothesized that a potential cleavage site exists somewhere within this bridge peptide region (Fig. 6.5B). In addition, I also calculated the size of the N-lobe peptide of goldfish transferrin (GenBank Accession# AAK92216) by adding the masses of each individual amino acid starting from the N-terminal methionine (M) until the approximate mass of 34, 000 was reached (i.e. equivalent to the mass of N-lobe determined by MALDI-TOF analysis; Fig. 5.19). Similar analysis was performed by calculating the individual masses of the amino acids starting from the C-terminal end of goldfish transferrin. As shown in Fig. 6.6, the calculated masses of the N- and C-lobes of goldfish transferrin resulted in the prediction of a cleavage site within the interdomain bridge peptide. More specifically, I estimated the cleavage site to be between amino acids 338 to 342 (I, Q, W, C, and T) of the reported goldfish amino acid sequence. These amino acids were also located within the putative peptide bridge region separating the lobes of goldfish transferrin (Fig. 6.6). It should be noted, however, that this calculation was only an estimation of the potential cleavage site. In addition, there have been several goldfish transferrin isoforms recently submitted to GenBank (Accession# AAL57603, AAM90973, AAM90971, AAM90972, AAL57602, AAK92216, AAM90970) making a precise determination of the cleavage site difficult. Protein alignments of these isoforms revealed a very high degree of amino acid similarity ranging from 85.9 to 97.3% when compared to the goldfish transferrin amino acid sequence that was originally used for the calculation of the potential cleavage site (AAK92216). There was also >95% amino acid conservation within the putative bridge peptide for all of the goldfish transferrin isoforms examined (Fig. 6.5B).

Amino acid alignments of transferrin from different species (i.e. goldfish, carp, trout, frog, chicken, bovine, and human) were then performed and as shown in Fig. 6.6, the estimated cleavage site that was calculated for goldfish transferrin (in box), was present within the predicted bridge peptide region (outlined). Interestingly, all of the different transferrin species analyzed contained a conserved tryptophan (W) and cysteine (C) residue within the bridge peptide, which may represent a conserved cleavage site for the conversion of the full-length protein into active NO-inducing fragments.

6.2.5 Detection of goldfish transferrin open reading frame (ORF) and design of construct primers for recombinant protein expression

There are several different isoforms of goldfish transferrin submitted to the GenBank database, which share >95% amino acid identity throughout the native molecule and >95% homology within the predicted bridge peptide region. Using the cDNA sequence derived from GenBank Accession# AAK92216 (which shared the highest homology to the sequence of the PCR products generated from goldfish liver cDNA templates using goldfish transferrin-specific PCR primers), the ORF of goldfish transferrin was predicted using GenTool DNA analysis software. From this cDNA, it was determined that the goldfish transferrin ORF was 2012 bp, which encoded a 671 amino acid protein. This information was subsequently used for designing PCR primers for generating expression constructs after cloning into the prokaryotic expression vector pET100/D-TOPO® or the eukaryotic expression vector pIB/V5-His-TOPO®, which were both purchased from Invitrogen. A map of the PCR primers used for creating goldfish transferrin full-length, N-lobe, and C-lobe constructs was shown in Figure 6.7. The sequence of the individual primers was reported in Table 1.1 (Chapter 2). Note: all constructs were sequenced prior to proceeding with recombinant expression to verify that they were cloned into the expression vectors in the correct orientation, were 'in frame', and to ensure that the correct gene was cloned.

6.2.6 Cloning of goldfish transferrin into pET100/D-TOPO® and expression of recombinant proteins in *E. coli*

6.2.6.1 Cloning and verification of prokaryotic expression constructs

Using the prokaryotic expression primers (Table 1.1, Chapter 2), PCR products were generated that correlated with the expected sizes for goldfish transferrin full-length (~2000 bp), N-lobe (~1000 bp), and C-lobe (~1000 bp) (Fig. 6.8A). Each PCR product was subsequently cloned into the pET100/D-TOPO® expression vector and positive clones were identified by randomly selecting 10 clones from each construct and performing colony PCR using the vector specific primers T7 and T7 reverse. Colonies containing the correct insert size were used for isolation of plasmid DNA and the constructs were subsequently verified by DNA sequencing also using the vector specific primers T7 and T7 reverse. Furthermore, constructs were digested with Nhe 1 and Sac 1, which are restriction enzymes that cut at sites that flank the multiple cloning site of the pET100/D-TOPO® expression vector (www.invitrogen.com). As shown in Fig. 6.8B, a single cut with Nhe 1 resulted in the detection of the plasmid (5764 bp) plus the size of the various cloned inserts. Therefore, pET100/D-TOPO® containing full-length transferrin was ~7764 bp whereas the vector with N- or C-lobe fragments was ~6764 bp. pET100/D-TOPO® cut with both Nhe 1 and Sac 1 resulted in the release of the cloned fragments further verifying the expression constructs.

6.2.6.2 Induction of recombinant protein expression and purification of the recombinant proteins

The pET100/D-TOPO® prokaryotic expression vector uses elements from bacteriophage T7 to control expression of heterologous genes in *E. coli*. The BL21 Star™ (DE3) *E. coli* strain used for recombinant protein expression carried the DE3 bacteriophage lambda lysogen, which contained a lac construct consisting of the lacI gene encoding the lac repressor, the T7 RNA polymerase gene under control of the lacUV5 promoter, and a small portion of the lacZ gene. Therefore, addition of the inducer, isopropyl β-D-thiogalactoside (IPTG) allowed for expression of T7 polymerase from the lacUV5 promoter. As shown in Fig. 6.9, induction of transfected BL21 Star™ (DE3) cells with 0.1 mM IPTG induced maximal levels of protein expression between 2-4 h post-induction. Figure 6.9 demonstrates the detection of recombinant goldfish transferrin full-length and the C-lobe fragment (Fig. 6.9A and 6.9B, respectively) using Western blot and an anti-HisG antibody. The production of recombinant goldfish transferrin N-lobe fragment was also performed, and was found to be similar to C-lobe (data not shown). In addition, recombinant protein expression was slightly increased when the transfected *E. coli* were incubated in LB supplemented with 50 µg/ml carbenicillin (Fig 6.9). Detection of the recombinant proteins was possible due to the insertion of an N-terminal 6xHis-tag encoded by the expression vector. The same 6xHis-tag was also used for purification of the recombinant proteins as described below.

Under the conditions chosen for the expression of recombinant goldfish transferrin in *E. coli*, inclusion-bodies were formed, which contained the majority of the recombinant proteins (Fig. 6.10). The isolation of inclusion-bodies and subsequent solubilization of recombinant goldfish transferrin C-lobe using urea was shown in Figure 6.10. Following isolation from inclusion-bodies, recombinant proteins were renatured using reduced and oxidized glutathione (GSH and GSSG, respectively) as described in the Materials and Methods (Chapter 2). Renatured proteins were then further purified using nickel columns. Figure 6.11A, demonstrates the purity of recombinant goldfish transferrin following isolation from inclusion-bodies and renaturation as determined by SDS-PAGE and Coomassie blue staining. It was also evident from Figure 6.11A that goldfish transferrin full-length was produced in significantly lower amounts than either N- or C-lobe produced under identical conditions. Following application to HisTrap columns, the recombinant proteins containing an N-terminal 6xHis-tag were eluted with imidazole (Fig. 6.11B), dialyzed overnight vs. 4 L of 1xPBS, filter-sterilized, protein content determined, and stored at 4°C prior to use in biological assays.

6.2.6.3 Induction of NO production in goldfish macrophages by recombinant goldfish transferrin produced in *E. coli*

Induction of NO production in goldfish macrophages by recombinant transferrin was tested immediately following elution from the nickel columns prior to the removal of imidazole. Similar to other reports, contaminating imidazole significantly impaired NO production by goldfish macrophages [654]. As little as 4.7 mM imidazole potently inhibited the production of NO in activated goldfish macrophages (Fig. 6.12). Consequently, any potential NO-inducing activity of the recombinant proteins would most likely be abrogated by the presence of imidazole, which must be removed prior to assessing the biological activity of these recombinant proteins.

Following an overnight dialysis vs. 4L of 1xPBS, 150 and 300 ng of recombinant transferrin C- and N-lobes induced significant production of NO-production in goldfish macrophages when compared to unstimulated cells (Fig. 6.13A and Fig. 6.13B, respectively). Co-incubation of goldfish macrophages with recombinant goldfish C- or N-lobe transferrin fragments with 1 μg/ml LPS caused a significant induction of NO production at all concentration of recombinant proteins tested (i.e. 37.5 to 300 ng) when compared to cells stimulated with 1 μg/ml LPS alone. This effect was remarkably similar to goldfish macrophages treated with crude MAF and LPS, as indicated by the positive control. Conversely, the highest dose of full-length recombinant goldfish transferrin (i.e. 200 ng/well) tested failed to induce production of NO in goldfish macrophages, alone or in conjunction with 1 μg/ml LPS (e.g. 0.655 μM ± 0.252 and 2.182 μM ± .667, respectively; Note: the nitrite production induced by 1 μg/ml LPS alone was 2.218 μM ± 0.202).

Since the recombinant proteins were produced in *E. coli*, the likelihood of endotoxin contamination was high. In addition, the production of NO by goldfish macrophages could be induce by LPS alone, thus removal of potential endotoxin contamination was essential for correct interpretation of results. Following purification, 1 ml samples of the recombinant proteins were applied to Detoxi-Gel™ endotoxin removal columns. The endotoxin removal columns bind approximately 400-600 ng of LPS/mL of gel. After application to the columns, both recombinant goldfish transferrin N- and C-lobes were re-tested for NO-inducing activities (Fig.

6.14 and 6.15, respectively). The samples were also dialyzed following elution from the Detoxi-Gel™ columns overnight vs. 1xPBS to remove any potential sodium deoxycholate that may have co-eluted with the recombinant proteins. Sodium deoxycholate was used to regenerate the endotoxin removal columns prior to application of samples and the effects of this reagent on goldfish macrophage antimicrobial functions was unknown.

Following application to the endotoxin removal column, non-dialyzed recombinant goldfish transferrin N-lobe alone, at concentrations ranging from 37.5 to 300 ng, did not induce a significant increase of NO production in goldfish macrophages compared to unstimulated cells (Fig. 6.14A). However, combined treatments with recombinant N-lobe (37.5 to 300 ng) and LPS (1 μg/ml) produced significantly more NO than cells stimulated with LPS alone at all concentrations of recombinant protein tested (Fig. 6.14A). To determine whether contaminating sodium deoxycholate may be responsible for the failure of recombinant N-lobe alone to induce NO production, samples were dialyzed and re-tested. Dialyzed recombinant N-lobe alone exhibited a dose-dependent increase in NO production with the highest concentration of protein (i.e. 300 ng) inducing maximal activity (Fig. 6.14B). In conjunction with 1 μg/ml LPS, recombinant N-lobe (75-300 ng) induced significantly higher levels of NO production than cells stimulated with LPS alone, and this effect was dose-dependent (Fig. 6.14B). Therefore, following application of samples to Detoxi-Gel™ columns, it was essential to remove contaminating sodium deoxycholate.

Similar effects were observed with recombinant goldfish transferrin C-lobe (Fig. 6.15). Prior to removal of sodium deoxycholate, recombinant C-lobe alone at doses ranging from 37.5 to 300 ng induced significant production of NO in goldfish macrophages when compared to untreated controls, however, the effect was not dose-dependent (Fig. 6.15A). In combination with LPS (1 μg/ml), 37.5 to 300 ng of non-dialyzed recombinant C-lobe induced significantly more NO production than cells treated with LPS alone and as similarly observed for non-dialyzed N-lobe, this effect was not dose-dependent (Fig. 6.15A). Following the removal of contaminating sodium deoxycholate by dialysis, a dose-dependent induction of NO in goldfish macrophages by recombinant goldfish transferrin C-lobe alone or in combination with 1 μg/ml LPS was observed (Fig. 6.15B). Finally, using the limulus amebocyte lysate assay, it was determined that following application to the endotoxin removal columns, recombinant samples contained <0.250 EU/ml (<0.025 pg/μl) of endotoxin such that the maximal amount of endotoxin contamination that could have been presented to the macrophages was 0.625 pg/well. This value is ~40 000x lower than the control levels of LPS (1 μg/ml or 25 ng/well) that I used to stimulated goldfish macrophages.

6.2.6.4 Induction of NO production in murine macrophages by recombinant goldfish transferrin produced in *E. coli*

Production of NO by the murine macrophage-like cell line P388D.1 was observed following treatment with recombinant goldfish transferrin C-lobe (Fig. 6.16). In the presence of LPS-inhibitor polymixin B (PMB) sulphate, only the highest concentration of recombinant C-lobe tested (i.e. 300 ng) demonstrated a significant reduction in NO production (Fig. 6.16A). However, this PMB-mediated reduction was minimal (~30%) compared with the complete inhibition of NO production induced by LPS (100 or 10 ng/ml) in conjunction with 10 μg/ml PMB sulphate (Fig. 6.16B), indicating that the NO-inducing activity of the recombinant protein was not exclusively due to endotoxin contamination. Furthermore, recombinant full-length goldfish transferrin failed to induce NO production at all concentrations examined (Fig. 6.16B) and this recombinant protein was prepared under identical conditions as the active recombinant C-lobe.

Induction of NO production by treatment with recombinant goldfish transferrin was also observed in primary murine macrophages. Bone marrow-derived murine macrophages (BMDM) were treated with low and high concentrations of LPS (i.e. 1000 and 10 ng/ml) or low and high concentration of recombinant goldfish transferrin C-lobe (i.e. 300 and 37.5 ng). Both low and high concentration of LPS or recombinant C-lobe induced NO production by these cells, however, in the presence of 10 μg/ml of the LPS-inhibitor PMB sulphate, only LPS-treated cells demonstrated a significant reduction of NO production (Fig. 6.17). These results suggested that the induction of NO production by treatment with recombinant C-lobe in BMDM was not due to the presence of contaminating endotoxins.

6.2.7 Cloning of goldfish transferrin into pIB/V5-His-TOPO® and expression of recombinant proteins in insect cells

6.2.7.1 Cloning and verification of eukaryotic expression constructs

Using the eukaryotic expression primers (Table 1.1), PCR products were generated that correlated with the expected size for goldfish transferrin full-length (i.e. ~2000 bp; Fig. 6.18A). The PCR product was subsequently cloned into the pIB/V5-His-TOPO® expression vector and positive clones were identified by randomly selecting 10 clones and performing colony PCR using the vector specific primers OpIE2 forward and reverse (Fig. 6.18B). Colonies containing the correct insert size were used for isolation of plasmid DNA and the constructs were subsequently verified by DNA sequencing using the vector specific primers OpIE2 forward and reverse. Furthermore, constructs were digested with BamH1 and Not 1, which are cut-sites that flank the multiple cloning site of the pIB/V5-His-TOPO® expression vector (www.invitrogen.com). As shown in Fig. 6.19A, pIB/V5-His-TOPO® cut with the restriction enzymes BamH1 and Not 1 resulted in the release of the cloned fragments, which correlated with the sizes of the PCR product originally cloned into the expression vector.

6.2.7.2 Recombinant protein expression in insect cells and purification of the recombinant proteins

The pIB/V5-His-TOPO® expression vector allowed for the expression of recombinant proteins in insect cell lines transiently or stably. Stable cell lines were selected using the Blastacidin resistance gene. Recombinant protein expression was achieved by using the baculovirus immediate-early promoters, which utilized the host cell transcription machinery and did not require viral factors for activation. Both the Opie-2 and Opie-1 promoters were from the baculovirus *Orgya pseudotsugata* multicapsid nuclear polyhedrosis virus (OpMNPV). The virus' natural host is the Douglas fir tussock moth; however, the promoters also allowed for protein expression in several other insects, and I chose the cell-line Sf9 from *Spodoptera frugiperda* for recombinant expression of goldfish transferrin. Recombinant protein expression was detected using an anti-V5 monoclonal antibody that detects proteins that contain a C-terminal V5 epitope encoded by the pIB/V5-His-TOPO® expression vector. Following 48 h of cellfectin-mediated transfection of Sf9 cells with the expression construct, both cell supernatants and lysates were examined by Western blot for the detection of recombinant proteins (Fig. 6.19B). Recombinant goldfish full-length protein was detected in supernatants and cellular lysates following transfection of the insect cell line. Following selection with Blasticidin, the stable polyclonal cell lines continued to produce recombinant goldfish transferrin (Fig. 6.19C) as determined by Western blot analysis.

Recombinant goldfish transferrin produced using the pIB/V5-His-TOPO® expression vector also contained a C-terminal 6xHis-tag, which was subsequently used for purification of the protein using a nickel-agarose (NiNTA) column. Recombinant full-length goldfish transferrin bound to the NiNTA column and was subsequently eluted with 125 mM imidazole (Fig. 6.20). The purified recombinants were then dialyzed overnight vs. 1xPBS, filter-sterilized, and stored at 4°C prior to use in biological assays.

6.2.7.3 Biological activity of recombinant goldfish transferrin produced in insect cells

Incubation of goldfish macrophages with recombinant full-length goldfish transferrin (1 μg) produced in Sf9 cells did not induce the production of NO (Fig. 6.21A). The cells tested were responsive to activation stimuli since treatment with MAF and LPS induced NO production in these cells, and as demonstrated earlier, treatment with elastase also induced the production of NO in these cells. Although recombinant goldfish full-length transferrin did not induce the production of NO, combined treatments of recombinant goldfish transferrin with elastase induced significantly more NO production in goldfish macrophages than elastase-treated cells alone and this effect was significantly inhibited by addition of 10 mM PMSF (Fig. 6.21A), which also significantly inhibited NO production by MAF and LPS activated macrophages. I also tested two other serum proteins that have been shown to activate macrophages in mammals. Treatment of goldfish macrophages with 10 μg human fibrinogen or fibronectin alone did not exhibit any activity (Fig. 6.21B). However, in combination with elastase, a significant increase in NO production was observed, which was significantly greater than macrophages incubated with elastase alone (Fig. 6.21B). As observed with recombinant goldfish transferrin produced in insect cells, this increase in NO production induced by combined treatments of elastase and serum proteins was significantly inhibited by the addition of 10 mM PMSF (Fig. 6.21B).

As demonstrated earlier, elastase could cleave serum transferrin and commercially purchased transferrin into smaller fragments resulting in the NO-inducing activity of this serine protease (Fig. 6.3). Therefore, enhanced NO production by co-incubation of goldfish macrophages with elastase and recombinant goldfish transferrin produced in insect cells as well as fibrinogen or fibronectin may also be due to the cleavage of these proteins. As shown in Figure 6.22A, elastase cleaved all of these proteins into smaller fragments. In addition, the elastase-mediated induction of NO production was also observed in murine macrophages. Treatment of P388D.1 murine macrophage-like cells with 5 μg/ml of elastase significantly induced the cells to produce NO (Fig. 6.22B). Interestingly, combined treatments of recombinant full-length goldfish transferrin with elastase induced significantly more

NO in P388D.1 macrophages than elastase alone (Fig. 6.22B). This effect was also observed by combination of elastase and fibrinogen or fibronectin and as observed in goldfish macrophages, this effect was significantly abrogated by the addition of the enzyme inhibitor PMSF (Fig. 6.22B). Therefore, induction of NO production in macrophages by recombinant goldfish transferrin produced in insect cell was dependent on the appropriate cleavage of this protein. It also appears that following cleavage, other serum proteins exhibited similar effects (i.e. fibrinogen and fibronectin).

6.3 Discussion

Results of this chapter further confirmed that transferrin cleavage products induce the production of NO in goldfish macrophages. Furthermore, it appeared that this was a conserved mechanism of macrophage activation since recombinant goldfish transferrin induced NO production in both teleost and mammalian macrophages. I also demonstrated that transferrin from different species may contain a conserved cleavage site required for the activation of this molecule. The immunostimulatory transferrin fragments were originally purified from mitogen-stimulated goldfish kidney leukocytes as I have described in Chapter 5. Transferrin was most likely derived from the serum, which was used to supplement the cultures and not secreted by goldfish kidney leukocytes since they failed to express transferrin mRNA following mitogen stimulation. However, activated goldfish macrophages did express transferrin mRNA and may also produce this protein, which has been shown for mammalian macrophages [655-656].

Cleavage of transferrin was mediated by an unidentified enzyme(s) present in mitogen-stimulated goldfish kidney leukocyte supernatants and by enzymes released from damaged goldfish cells (i.e. granulocytes and macrophages). During the inflammatory response, cellular damage and lysis resulted in the release of intracellular contents due to the loss of membrane integrity [653] and necrotic cell death appears to be the primary cytotoxic mechanism following tissue injury. Cell death by necrosis (but not apoptosis) activates NF-κβ in viable fibroblasts, macrophages, and dendritic cells [657-658] and it is well accepted that cell death mediated by apoptosis or necrosis induced anti- and pro-inflammatory processes, respectively [653]. It has also recently been reported that intracellular contents from necrotic cells mediated NF-κβ activation through the activation of TLR-2 [658]. This represented a novel pathway for the induction of genes involved in inflammation and tissue-repair by an endogenous ligand derived from necrotic cells. However, the molecular identity of this endogenous necrotic cell factor remains unknown. Stimulation of goldfish kidney leukocytes with mitogens also caused a significant amount of cellular lysis/necrosis and supernatants derived from these preparations demonstrated potent pro-inflammatory properties (i.e. macrophage activation) and transferrin-cleaving activity. Lysed goldfish cells (i.e. granulocytes and macrophages) also contained enzymes with transferrin-cleaving activity. Therefore, in response to cellular damage caused by mitogen stimulation, the release of intracellular enzymes from lysed goldfish cells resulted in the cleavage of transferrin into smaller fragments. These endogenous transferrin fragments then provided an activation signal for macrophages. Thus, it is possible that one of the potential molecules responsible for the pro-inflammatory activities of necrotic cell death is the release of intracellular enzymes. These enzymes may be responsible for cleaving endogenous proteins (i.e. transferrin), which then provide a signal for the induction of innate immune responses mediated through sentinel immune receptors (e.g. TLR).

One of the enzyme(s) responsible for the cleavage of goldfish transferrin is elastase. The incubation of goldfish macrophages with elastase induced a significant increase in the production of NO, which correlated with the generation of transferrin cleavage products. Western blot analysis of normal goldfish serum demonstrated the presence of a single transferrin band (~70 kD), however, following elastase treatment transferrin fragments (~30-50 kD) were detected. In addition, elastase mediated the cleavage of two other serum-derived proteins (i.e. fibrinogen and fibronectin) resulting in the generation of fragments, which correlated with an enhancement of NO production in goldfish macrophages. The transferrin-cleaving activities (or cleavage of other endogenous serum-derived proteins) by elastase may explain why goldfish macrophages treated with this enzyme produced NO. Interestingly, a similar effect was observed in elastase-treated murine macrophages.

Elastase is a serine protease with broad substrate specificity that is produced by activated immune cells, and degrades extracellular matrices to facilitate the migration of these cells [659-663]. This degradation is tightly controlled by serum- and cell-derived inhibitors [659, 664-666], which may explain why relative large amounts of enzyme (i.e. 1-10 μg) are required to induce the production of NO in goldfish and murine macrophages *in vitro*. Goldfish macrophages activated with MAF and LPS exhibited a significant reduction in NO production following treatment with the enzyme inhibitor PMSF. Therefore, the physiological concentrations of endogenous enzymes generated by activated macrophages, were susceptible to PMSF-mediated inactivation, resulting in a dramatic inhibition of NO production. This suggested a key role for enzymes in the induction of NO production in goldfish macrophages. This finding was particularly interesting since in response to fungal infections, activation of *Drosophila* immune responses requires the initiation of a blood serine protease cascade [667], which can be inhibited by a serine protease inhibitor (serpin), which has potent effects on many proteases including elastase [668]. Furthermore, LPS-mediated activation of NF-κβ in murine monocytes was blocked by the serine protease inhibitor antithrombin III [157]. These results suggested that in response to pathogens, activation of extracellular serine proteases in invertebrates and vertebrates was required for initiation of immune responses, which was subsequently controlled by protease inhibitors.

During an inflammatory response, secretion of human leukocyte elastase was also responsible for the pathogenesis during a variety of inflammatory diseases. This includes bronchiectasis, cystic fibrosis, acute respiratory distress syndrome, bronchitis, and emphysema [669-670]. These diseases are primarily the consequence of the extensive proteolysis of extracellular matrix molecules such as collagens, fibronectin, laminin, proteoglycans, and elastin [671-672]. There was also a link between the presence of elastase, transferrin cleavage products, and tissue damage during inflammation in the lung. For example, a number of neutrophil and bacteria-derived proteases have been reported to alter the physiochemical nature of transferrin and lactoferrin at sites of *Pseudomonas aeruginosa* infection [673-674]. Furthermore, *P. aeruginosa* secretory products, such as elastase, cleaved both transferrin and lactoferrin into smaller protein fragments, which was believed to enhance the removal of iron from these proteins by pyoverdin [673-674]. Protease-cleavage of transferrin was believed to contribute to *Pseudomonas*-associated tissue injury by increasing iron availability to the pathogen and also increasing the potential for local hydroxyl radical formation, which can augment endothelial cell injury [675-677]. The binding of iron to transferrin limits its involvement in the generation of hydroxyl radical via the Haber-Weiss reaction [678] and this property may allow transferrin to function as an antioxidant *in vivo* [679-680]. Consequently, cleavage of iron-binding proteins at infection sites by both exogenous and endogenous derived proteases (i.e. elastase) augmented tissue damage by allowing iron to participate in the generation of toxic radicals. This process was particularly important in individuals suffering from cystic fibrosis and other forms of chronic lung disease as cleaved transferrin fragments have been identified in the bronchoalveolar fluids of these patients [674].

It is also possible that the uncontrolled degradation of transferrin (and other endogenous proteins) during lung diseases can exacerbate tissue damage following recognition by macrophages causing them to release ROI and RNI. This mechanism has never been explored, however, another endogenous lung protein has recently been identified to exhibit immunostimulatory activity, which involves the innate immune receptor TLR-4 [152]. Lung surfactant protein-A (SP-A) is a member of the collectin family and has been shown to enhance macrophage chemotaxis, phagocytosis, immune cell proliferation, expression of cell surface receptors, and production of cytokines [681-685]. While the lipid components of lung surfactants exhibit anti-inflammatory effects, the hydrophilic surface protein, SP-A, has been reported to exhibit both immunostimulatory and immunosuppressive activity [686]. It is believed that lung injury and/or infections disrupt the balance between the inhibitory and stimulatory influences of this protein resulting in tissue injury [687]. As such, increased levels of SP-A have been detected in pulmonary lavages from patients with pneumonitis and asbestosis [688] and their recognition by TLR-4 on the surface of lung macrophages may cause the release of pro-inflammatory cytokines and toxic reactive intermediates. Interestingly, transferrin-cleavage products are not present in the lungs of healthy patients and their presence during lung disease may also influence macrophage functions in a similar manner as SP-A. These potential mechanisms in lung infections and other inflammatory diseases remain to be determined.

In this chapter, I have also demonstrated that recombinant goldfish transferrin N- and C-lobe fragments exhibited potent NO-inducing activity. Recombinant transferrin has been successfully produced using both prokaryotic [527, 689-690] and eukaryotic [691-692] expression systems. The majority of studies use recombinant transferrin to further characterize its iron-binding properties [693], to study the interactions of transferrin with pathogen receptors [487], and to develop a novel approach for the biological delivery of peptide drugs [694]. To my knowledge, a role for recombinant transferrin as an endogenous activator of an immune responses has not been reported. My results reinforced the hypothesis that transferrin is an endogenous activator of macrophages, and that cleaved transferrin fragments represent a highly conserved pro-inflammatory signal that is potentially recognized by highly conserved family of innate immune receptors.

Transferrin represents an excellent candidate for an endogenous ligand of innate immune receptors since it is ubiquitously distributed throughout the body (i.e. a serum protein), is highly conserved throughout evolution, and must be degraded into smaller fragments for induction of macrophage activation [477-478]. It is also possible that transferrin fragments not only activate fish macrophages, but may also be recognized by mammalian macrophages as a highly conserved pro-inflammatory signal.

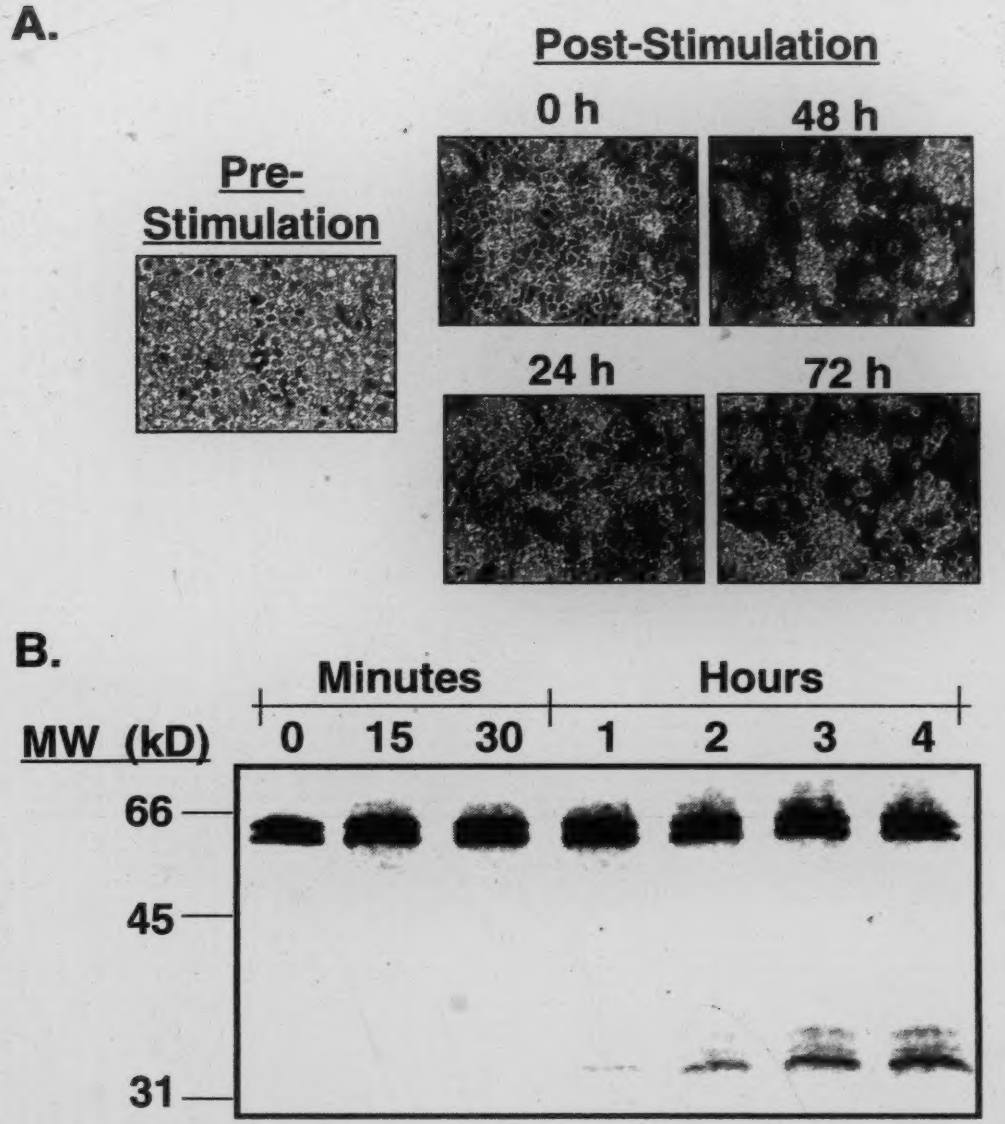


Figure 6.1. Detection of transferrin-cleaving activity in mitogen-stimulated goldfish kidney leukocyte supernatants. Goldfish kidney leukocytes were isolated, seeded into 75 cm^2 tissue culture flasks (4x10^6 cells/ml), and stimulated with mitogens. At the times indicated, the cultures were examined microscopically (A). Supernatants collected after 72 h post-stimulation were incubated with 2.5 μg of transferrin for 0, 15, 30 minutes, and 1, 2, 3, and 4 h and then examined by Western blot for transferrin-cleaving activity using the polyclonal anti-NO-inducing factor antibody (8C2) (B).

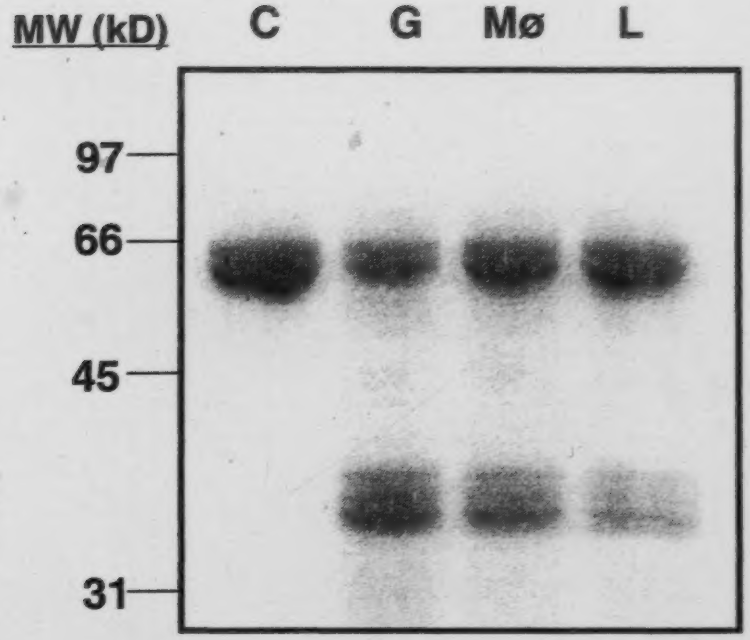


Figure 6.2. Detection of transferrin-cleaving activity in supernatants from lysed goldfish cells. Lysates prepared following freeze-thaw of various goldfish cells ($5x10^6$) (i.e. goldfish kidney granulocytes (G), goldfish macrophages (Mø), and goldfish kidney leukocytes (L)) were tested for the presence of transferrin-cleaving activity by incubating the lysates with 2.5 µg of transferrin for 4 h. Detection of transferrin-cleavage products was performed by Western blot analysis using the anti-NO-inducing factor polyclonal antibody (8C2). Control (C) represents incubation of transferrin (2.5 µg) in medium for 4 h prior to Western blot analysis.

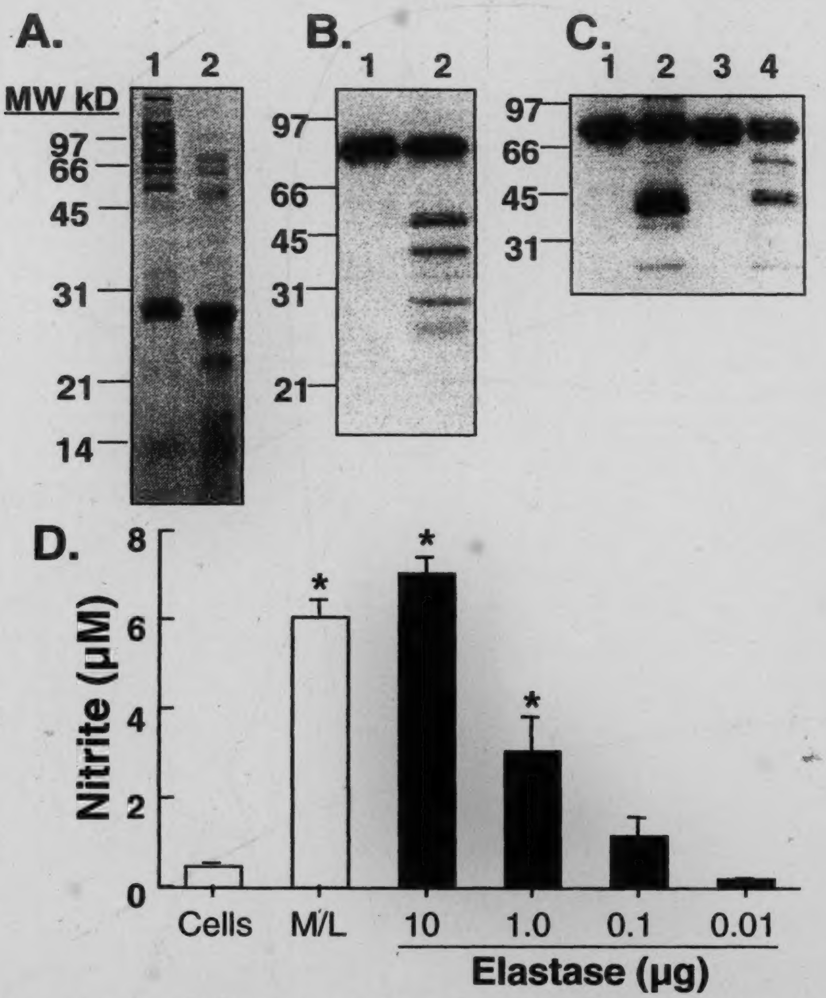


Figure 6.3. Generation of transferrin cleavage products and induction of NO production by elastase. Goldfish serum (0.1 µl) was incubated alone (Lane 1) or with 2 µg of elastase (Lane 2) for 2 h prior to separation by reducing SDS-PAGE and staining with Coomassie blue (A) or transfered to nitrocellulose membranes and probed with anti-carp transferrin polyclonal antibody (9AG7) (B). Generation of transferrin cleavage products was also determined by incubating 2.5 µg of bovine transferrin with 2 µg of elastase for 2 h prior to examination by Western blot using 8C2 polyclonal antibody (C); (Lane 1, bovine holo-transferrin alone. Lane 2, bovine holo-transferrin + elastase. Lane 3, bovine apo-transferrin alone. Lane 4, bovine apo-transferrin + elastase). Induction of NO production in goldfish macrophages by elastase treatment was assessed by incubating macrophages ($5x10^4$ cells/well) with 0.01 to 10 µg of elastase for 72 h prior to determination of NO production using the Griess reaction (D). Each bar represents the mean ± SEM of triplicate cultures and the results are from a representative experiment of three independent experiments performed. Note: (+) cells activate with crude MAF (1:4) and LPS (1 µg/ml) and * $P<0.05$ compared with cells incubated in medium alone.

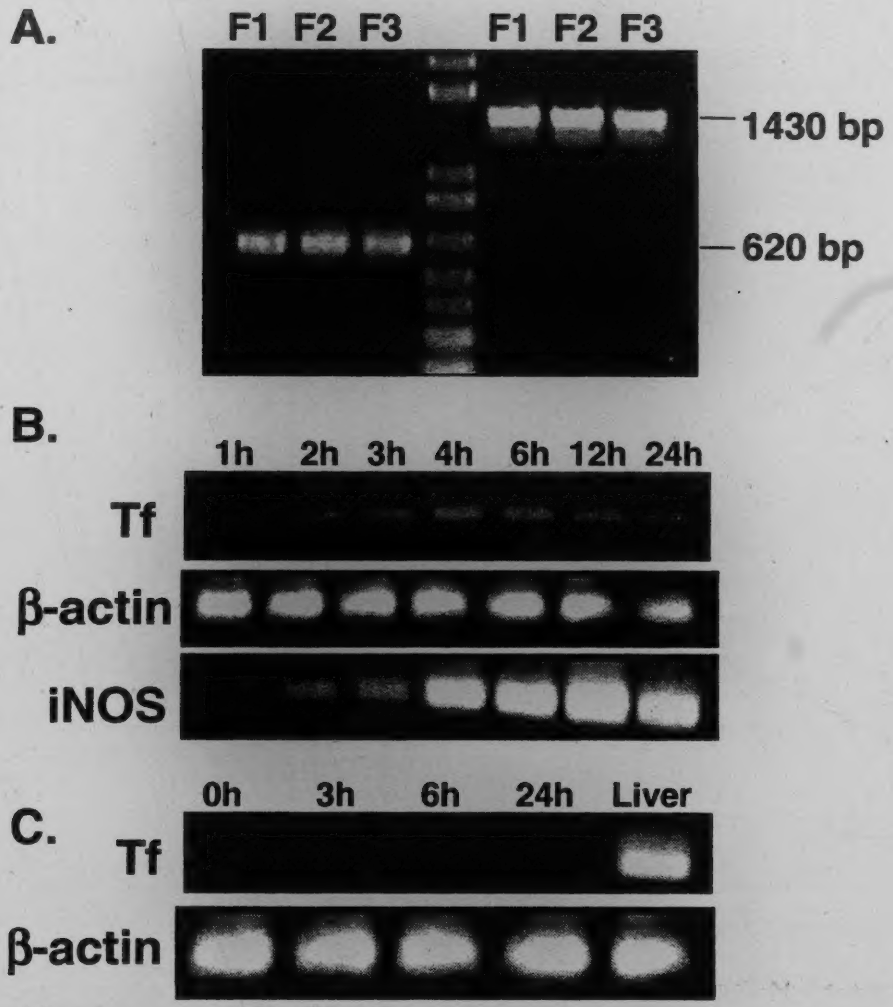
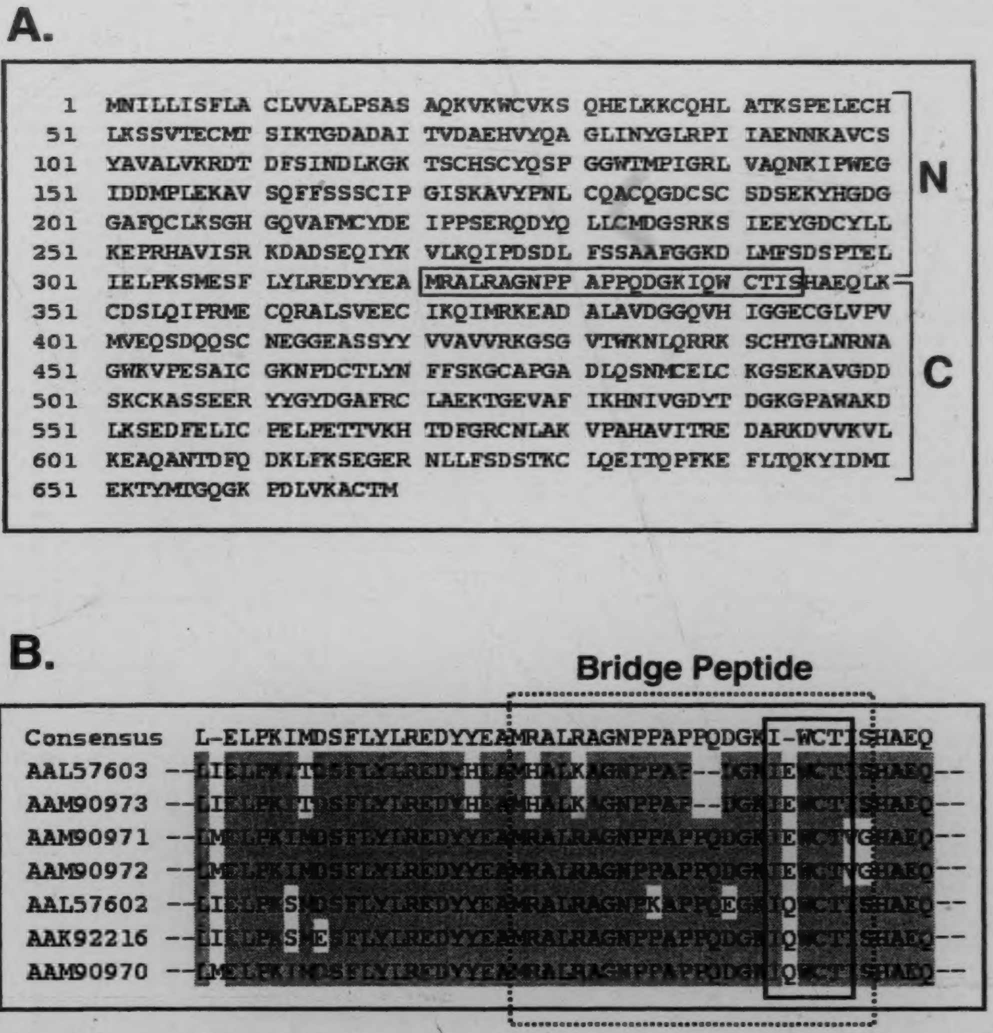


Figure 6.4. Goldfish transferrin (Tf) mRNA expression. RT-PCR was used to detect the expression of transferrin mRNA in goldfish liver (A), cultured macrophages (B), and kidney leukocytes (C). Fifty µg of liver tissue or 5 x 10^6 cultured macrophages or kidney leukocytes were used for total RNA extraction using TriZol reagent. Cultured macrophages were stimulated with MAF (1:4 final) and LPS (1 µg/ml) and kidney leukocytes were stimulated with mitogens for various time points prior to RNA extractions as described in the Materials and Methods chapter. Transferrin mRNA was expressed in liver tissue from the three fish examined (i.e. F1, F2, and F3) using two primers that generated products of 620 and 1430 bp (A), and was also expressed following activation of goldfish macrophages (B). Mitogen-stimulated kidney leukocytes did not demonstrate transferrin expression (C). Results are from a representative experiment of two independent experiments that were performed. DNA visualized following separation on a 1.2% agarose gel stained with EtBr



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  1  MNILLISFLA CLVVALPSAS AQKVKWCVKS QHELKKCQHL ATKSPELECH
 51  LKSSVTECMT SIKTGDADAI TVDAEHVYQA GLINYGLRPI IAENNKAVCS
101  YAVALVKRDT DFSINDLKGK TSCHSCYQSP GGWTMPIGRL VAQNKIPWEG
151  IDDMPLEKAV SQFFSSSCIP GISKAVYPNL CQACQGDCSC SDSEKYHGDG
201  GAFQCLKSGH GQVAFMCYDE IPPSERQDYQ LLCMDGSRKS IEEYGDCYLL
251  KEPRHAVISR KDADSEQIYK VLKQIPDSDL FSSAAFGGKD LMFSDSPTEL
301  IELPKSMESF LYLREDYYEA MRALRAGNPP APPQDGKIQW CTISHAEQLK
351  CDSLQIPRME CQRALSVEEC IKQIMRKEAD ALAVDGGQVH IGGECGLVPV
401  MVEQSDQQSC NEGGEASSYY VVAVVRKGSG VTWKNLQRRK SCHTGLNRNA
451  GWKVPESAIC GKNPDCTLYN FFSKGCAPGA DLQSNMCELC KGSEKAVGDD
501  SKCKASSEER YYGYDGAFRC LAEKTGEVAF IKHNIVGDYT DGKGPAWAKD
551  LKSEDFELIC PELPETTVKH TDFGRCNLAK VPAHAVITRE DARKDVVKVL
601  KEAQANTDFQ DKLFKSEGER NLLFSDSTKC LQEITQPFKE FLTQKYIDMI
651  EKTYMTGQGK PDLVKACTM
```

```
Consensus   L-ELPKIMDSFLYLREDYYEAMRALRAGNPPAPPQDGKI-WCTISHAEQ
AAL57603  --LIELPKTTDSFLYLREDYHEAMHALKAGNPPAP--DGKIEWCTISHAEQ--
AAM90973  --LIELPKTTDSFLYLREDYHEAMHALKAGNPPAP--DGKIEWCTISHAEQ--
AAM90971  --LMELPKIMDSFLYLREDYYEAMRALRAGNPPAPPQDGKIEWCTVGHAEQ--
AAM90972  --LMELPKIMDSFLYLREDYYEAMRALRAGNPPAPPQDGKIEWCTVGHAEQ--
AAL57602  --LIELPKSMDSFLYLREDYYEAMRALRAGNPKAPPQEGKIQWCTISHAEQ--
AAK92216  --LIELPKSMESFLYLREDYYEAMRALRAGNPPAPPQDGKIQWCTISHAEQ--
AAM90970  --LMELPKIMDSFLYLREDYYEAMRALRAGNPPAPPQDGKIQWCTISHAEQ--
```

Figure 6.5. Amino acid sequence of goldfish transferrin precursor derived from the cDNA sequence submitted to GenBank (Accession# AAK92216) (A). Shown is the amino acid sequence translated from a 2012 bp open reading frame, which encoded for a 671 amino acid protein. Goldfish transferrin consists of N-lobe (N) and a C-lobe (C) domains, which are separated by an interdmain peptide bridge (in box). Alignment of all the peptide bridge regions (dashed box) from all of the different goldfish transferrin isoforms submitted to GenBank is also shown, which includes the predicted cleavage site (in boxe) (B).

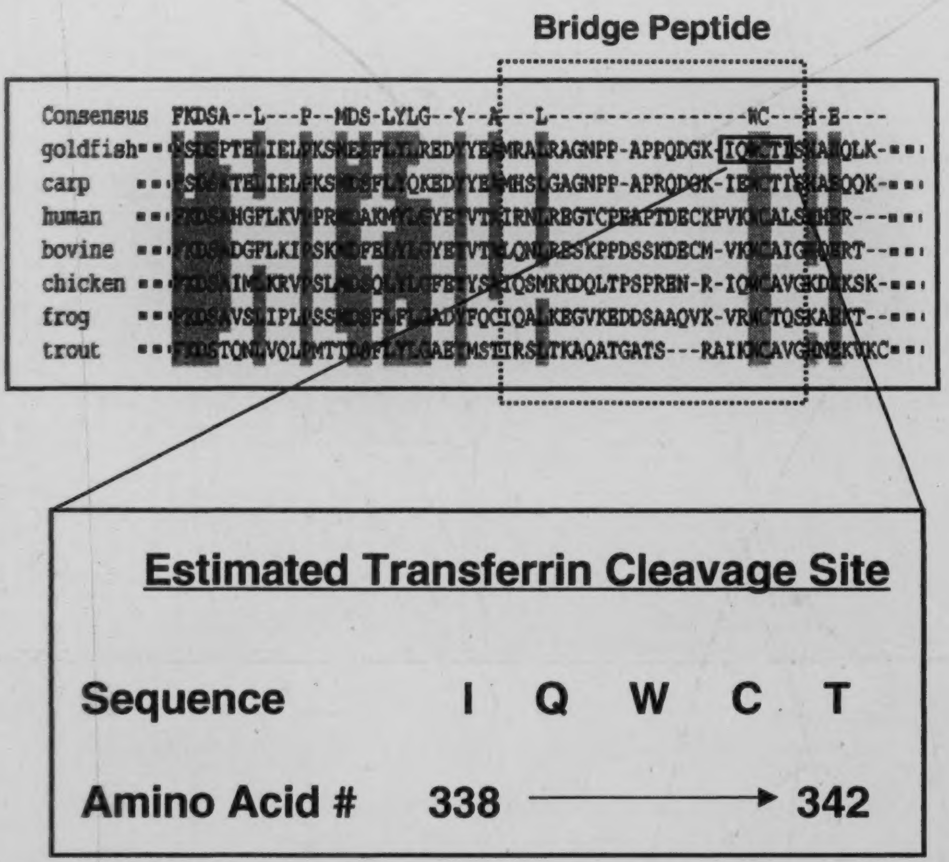
Bridge Peptide
Consensus FKDSA--L---P--MDS-LYLG--Y--A---L-----------------WC---H-E----
goldfish
carp
human
bovine
chicken
frog
trout
Estimated Transferrin Cleavage Site
Sequence
I Q W C T
Amino Acid #
338
342

Figure 6.6. Prediction of the goldfish transferrin cleavage site. Immunopurified goldfish transferrin fragment sizes were used to calculate a potential cleavage site. The molecular mass of the N-lobe and C-lobe, determined by MALDI-TOF analysis, were used to predict the cleavage site by adding the individual masses of each amino acid starting from the N-terminal methionine (M) or C-terminal amino acid as described in the materials and methods section. Shown is the predicted cleavage site between amino acids 338 and 342 of the goldfish transferrin precursor sequence (GenBank Accession AAK92216). The predicted cleavage was estimated to be within the bridge peptide that separates the two lobes of the transferrin molecule. Alignment of transferrin bridge peptide region from different transferrin species (dashed line), was performed to demonstrate the predicted cleavage site (box) for goldfish transferrin and the conservation of the tryptophan (W) and cysteine residues (C) within the predicted cleavage site of transferrin from different species.

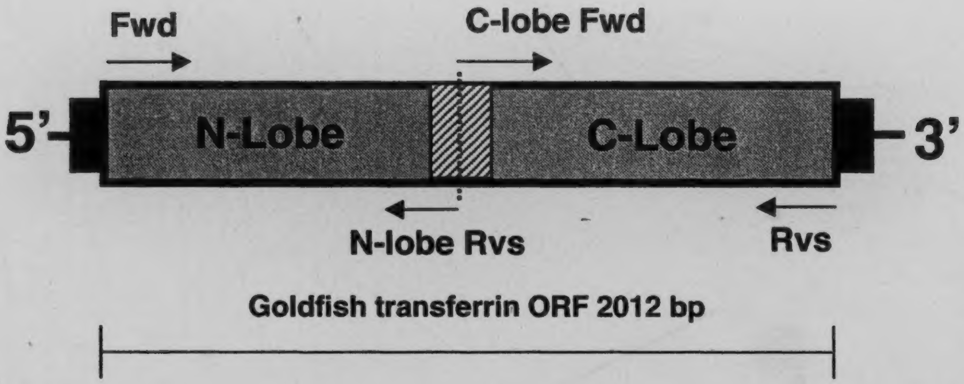

Figure 6.7. Map of primers used for generating PCR products for cloning into expression vectors. Using the goldfish transferrin cDNA ((GenBank Accession AAK92216), PCR primers were designed to amplify full-length transferrin, N-lobe, and C-lobe domains. In addition, N- and C-lobe fragments were generated by designing primers upstream (for N-lobe) and downstream (for C-lobe) of the predicted cleavage site (dotted line) within the bridge peptide region (thatched box). Forward primer (Fwd) in combination with the reverse primer (Rvs) were used to amplify full-length transferrin; The Fwd primer with the N-lobe Rvs primer were used to amplify the N-lobe fragment; and the C-lobe Fwd primer with the Rvs primer generated C-lobe fragment. See Table 1.1 (Chapter 1) for the specific nomenclature and nucleotide sequences of all primers used throughout this thesis.

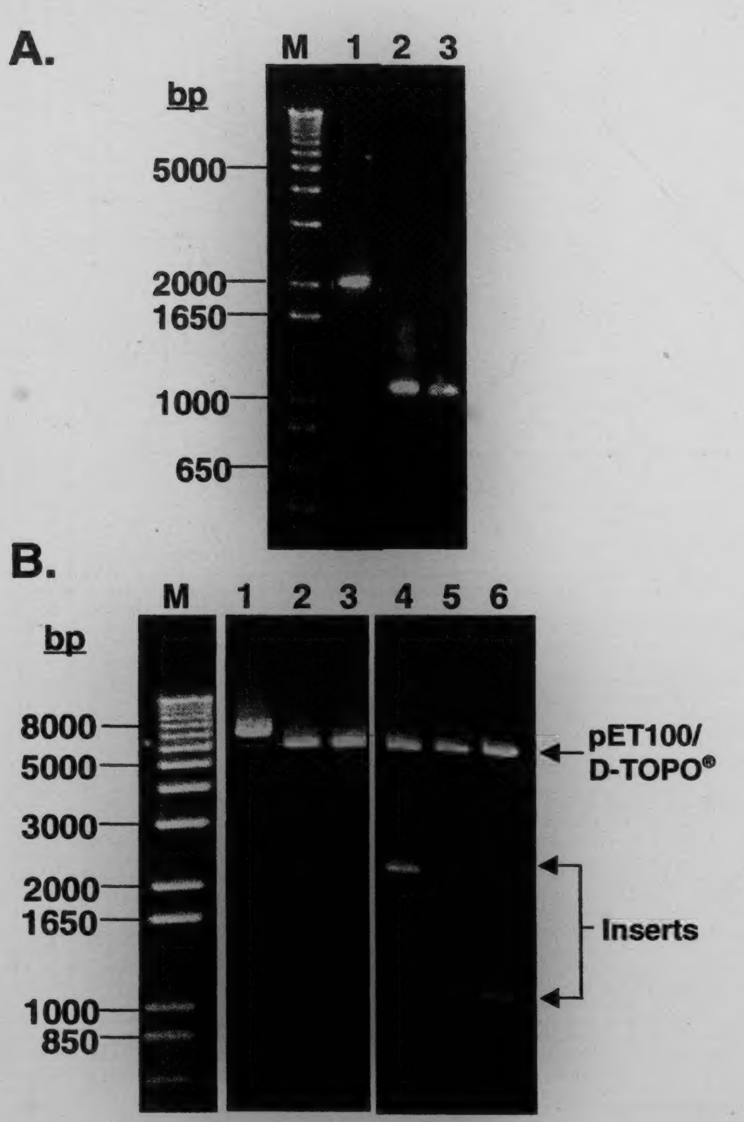

Figure 6.8. RT-PCR and cloning of goldfish transferrin into the prokaryotic expression vector pET100/D-TOPO®. RT-PCR was performed using the goldfish transferrin expression primers and goldfish liver cDNA was used as a template (A). Lane M, 1 Kb Plus DNA Ladder (Invitrogen); Lane 1, full-length transferrin; Lane 2, transferrin N-lobe; Lane 3, transferrin C-lobe. PCR products were subsequently cloned into the pET100/D-TOPO® expression vector and inserts detected by digestion with Nhe 1 and Sac 1 (B). Lane M, 1 Kb Plus DNA Ladder (Invitrogen); Lanes 1-3, Nhe 1 digested pET vector with full-length, N-lobe and C-lobe inserts, respectively; Lanes 4-6, Nhe 1 and Sac 1 digested pET100/D-TOPO® vector with full-length, N-lobe and C-lobe inserts, respectively. DNA visualized following separation on a 1.2% agarose gel stained with EtBr.

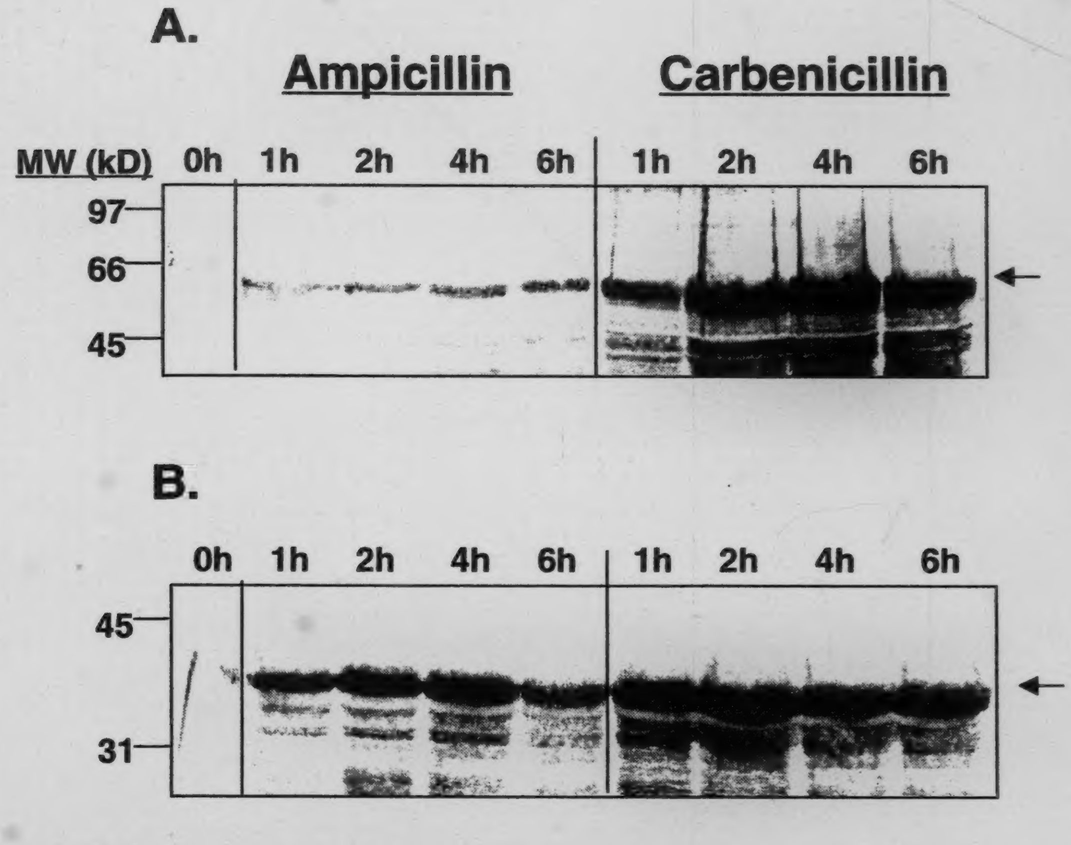

Figure 6.9. Detection of recombinant goldfish transferrin produced in *E. coli* by Western blot. Transformed BL21 Star (DE3) *E. coli* were grown in LB supplemented with either 100 µg/ml ampicillin or 50 µg/ml carbenicillin and induced with 0.1 mM IPTG. At times indicated, 500 µl sub-samples were removed, centrifuged at 14 000 rpm for 1 min., and supernatants aspirated. Both supernatants and cell pellets were resuspended in 2 X SDS-PAGE sample buffer and 1 µg of total protein was loaded onto a 12.5% reducing SDS-PAGE gel. Proteins were then transferred to nitrocellulose and the recombinant proteins visualized following an overnight incubation at 4°C with an Anti-HisG Antibody (1:5000). Shown is the detection of recombinant goldfish transferrin (full-length) (A), and C-lobe (B) in the cell pellets. Note recombinant protein expression was not detected in the supernatants (data not shown).

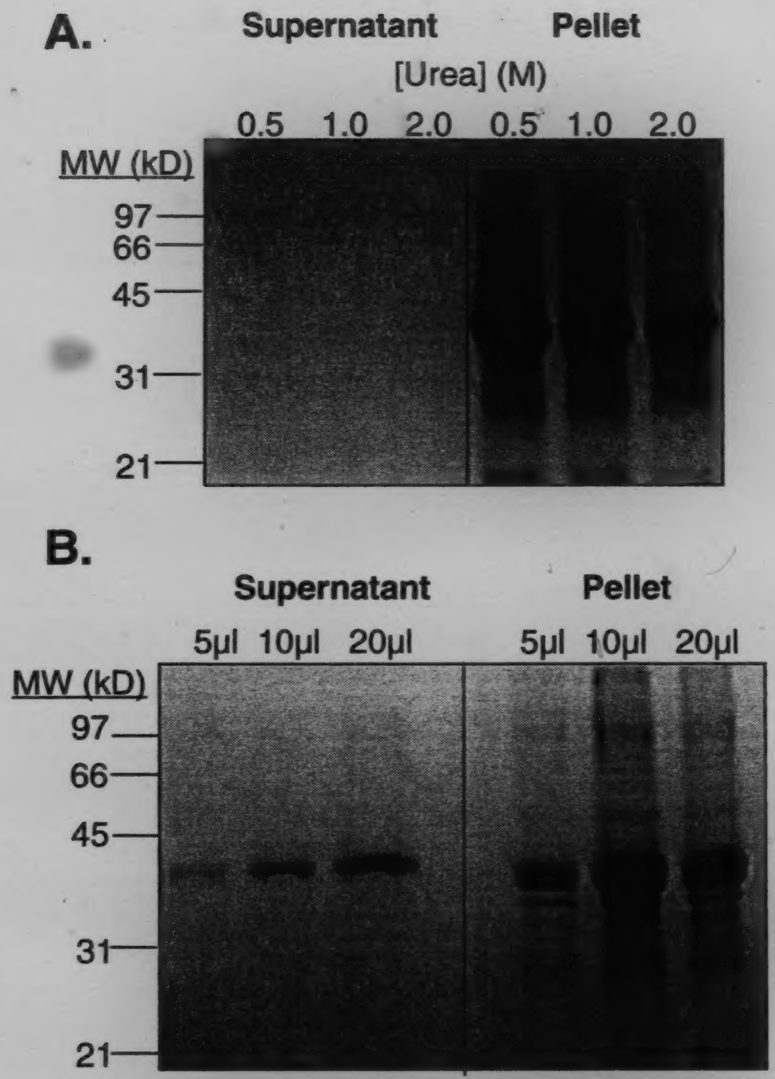

Figure 6.10. Isolation of inclusion-bodies and solubilization of recombinant goldfish transferrin. Following 2-4 h of induction with 0.1 mM IPTG induction, *E. coli* were harvested by centrifuging and the cell pellet lysed. Inclusion bodies were recovered as described in Materials and Methods chapter using 0.5-2.0 urea and following centrifugation and washing, inclusion bodies were detected in the insoluble pellet by reducing SDS-PAGE and Coomassie blue staining (A). Solublization of the inclusion-bodies and recovery of recombinant transferrin C-lobe from inclusion-bodies was performed as descrided in Materials and Methods, and following treatment with 8 M urea, recombinant protein was observed in the soluble supernatant and inclusion-bodies observed in the insoluble pellet (B).

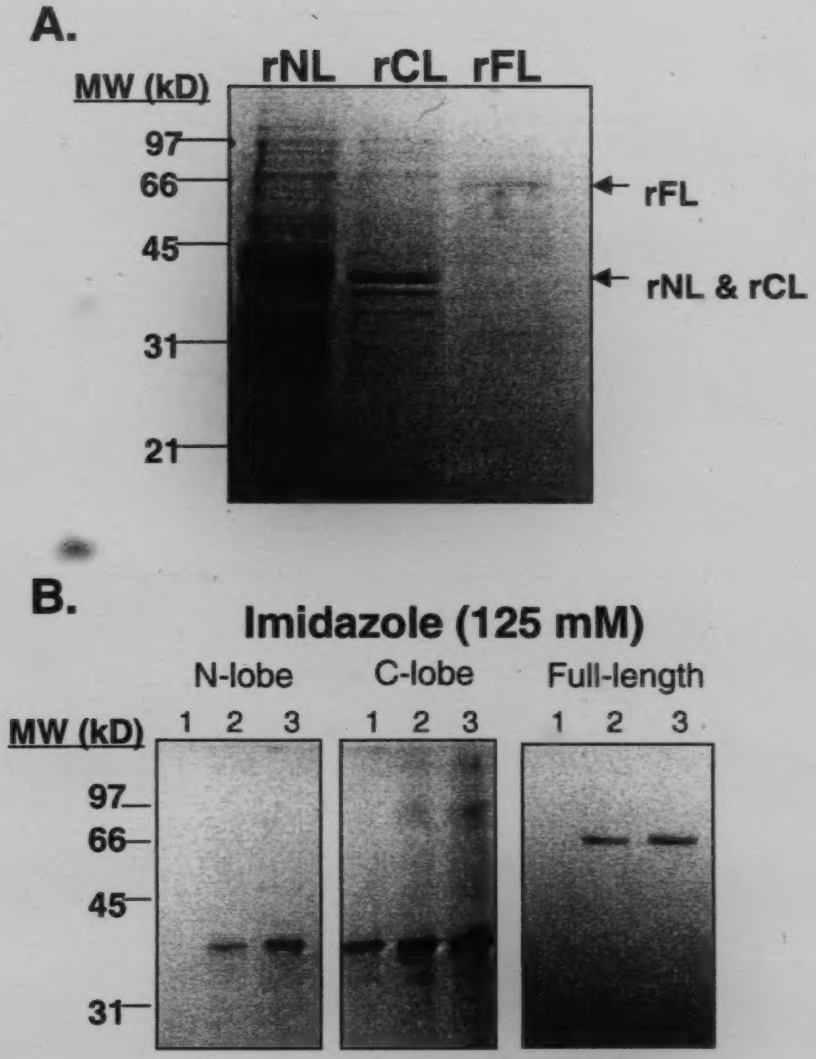

Figure 6.11. Purification of solubilized recombinant goldfish transferrin using HisTrap columns. Following recovery of recombinant proteins from inclusion bodies, samples contained a variety of contaminating proteins as determined by reducing SDS-PAGE and Coomassie blue staining (A). Further purification was performed using HisTrap columns and shown is a Coomassie blue stained SDS-PAGE gel of recombinant goldfish transferrin N-lobe, C-lobe and full-length following elution from the HisTrap column with 125 mM of imidazole. Numbers represent the elution fractions.

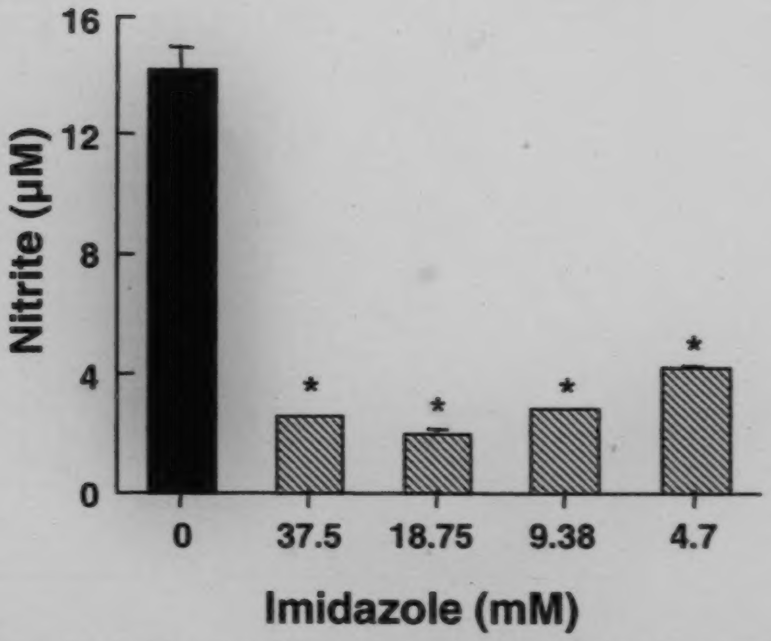

Figure 6.12. Effect of imidazole on the production of NO by goldfish macrophages. Goldfish macrophages were ($5x10^4$ cells/well) treated with various concentrations of imidazole in combination with MAF (1:4) and LPS (1 µg/ml) for 72 h at 20°C prior to determination of NO production using the Griess reaction. Control cells were activated with MAF (1:4) and LPS (1 µg/ml) in the absence of imidazole. Each bar represents the mean ± SEM of triplicate cultures and the results are from a representative experiment of three independent experiments performed. * $P<0.05$ vs. control NO production.

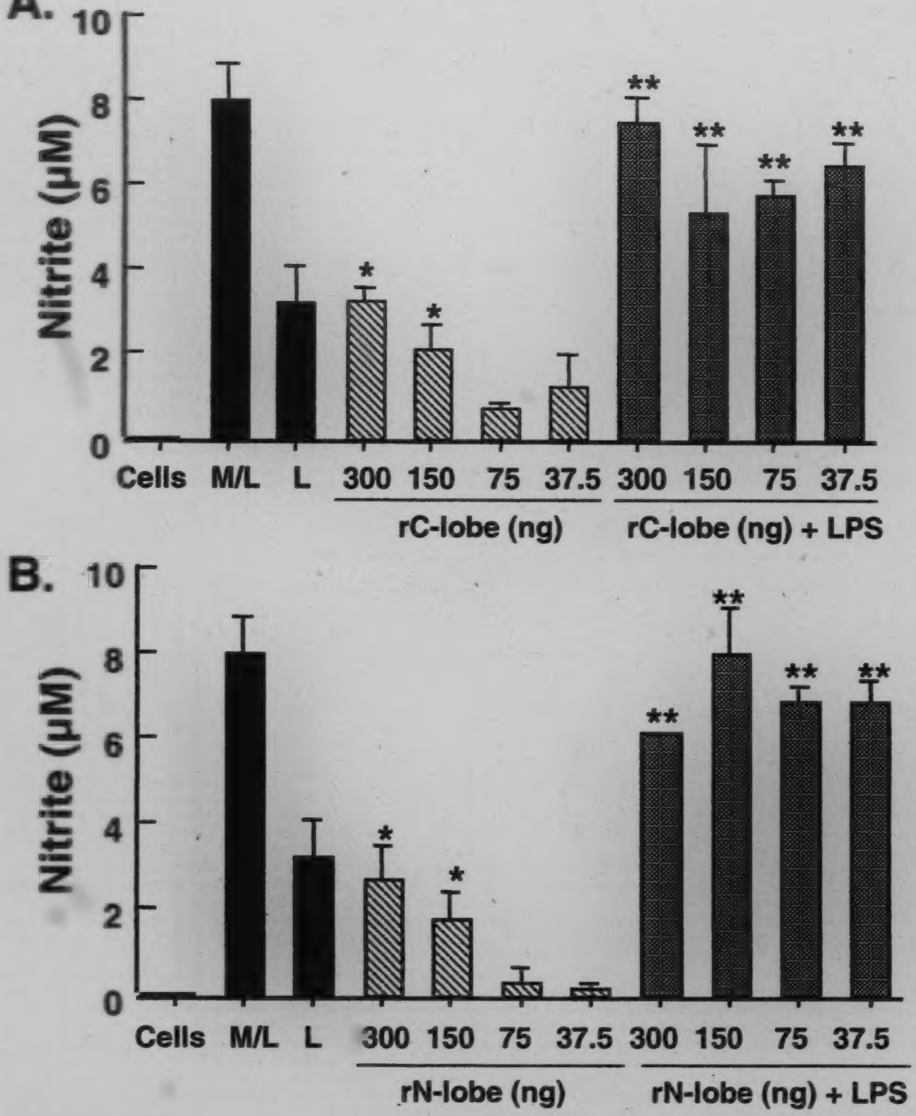

Figure 6.13. Induction of NO production in goldfish macrophages by treatment with recombinant transferrin C-lobe (A) and N-lobe (B) following removal of imidazole. Goldfish macrophages ($5x10^4$ cells/well) were treated with medium (cells), MAF (1:4) and LPS (1 µg/ml) (M/L), LPS (1 µg/ml) (L), recombinant protein alone (37.5 to 300 ng; thatched bars), or recombinant protein (37.5 to 300 ng) in conjunction with LPS (1 µg/ml; grey bars) for 72 h at 20°C prior to determination of NO production using the Griess reaction. Recombinant full-length (200 ng) did not induce NO production (see results). Each bar represents the mean ± SEM of triplicate cultures and the results are from a representative experiment of three independent experiments performed. * $P<0.05$ vs. unstimulated cells. ** $P<0.05$ vs. LPS stimulated cells.

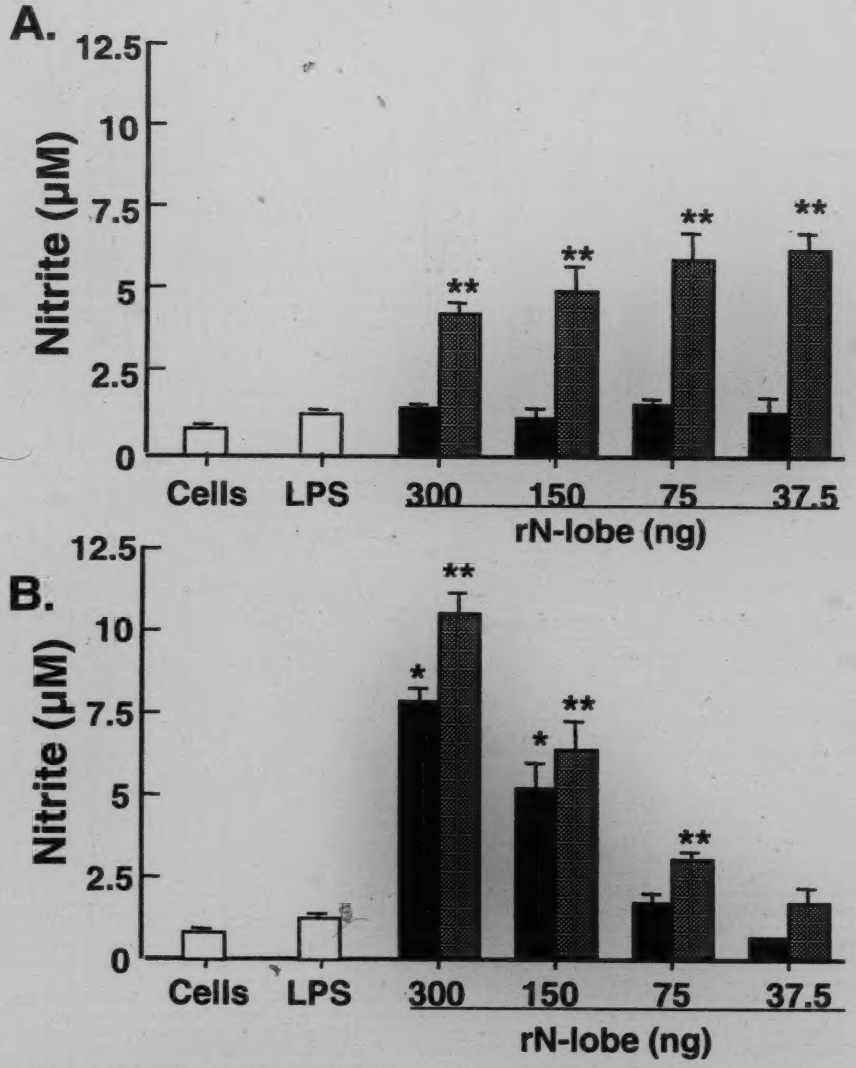

Figure 6.14. Induction of NO production in goldfish macrophages by treatment with recombinant transferrin N-lobe following application to a Detoxi-Gel™ LPS-removal column. One ml sample of purified recombinant protein was applied to a Detoxi-Gel™ column and incubated for 1 h at 4°C. Sample was then eluted from the column with 1xPBS and tested for NO-inducing activity immediately (A) or following an overnight dialysis vs. 4 L of 1xPBS (B). Goldfish macrophages (5×10^4 cells/well) were treated with medium (cells), LPS (1 μg/ml) (L), recombinant protein alone (37.5 to 300 ng; black bars), or recombinant protein (37.5 to 300 ng) in conjunction with LPS (1 μg/ml; grey bars) for 72 h at 20°C prior to determination of NO production using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and the results are from a representative experiment of two independent experiments performed. * $P<0.05$ vs. unstimulated cells. ** $P<0.05$ vs. LPS stimulated cells.

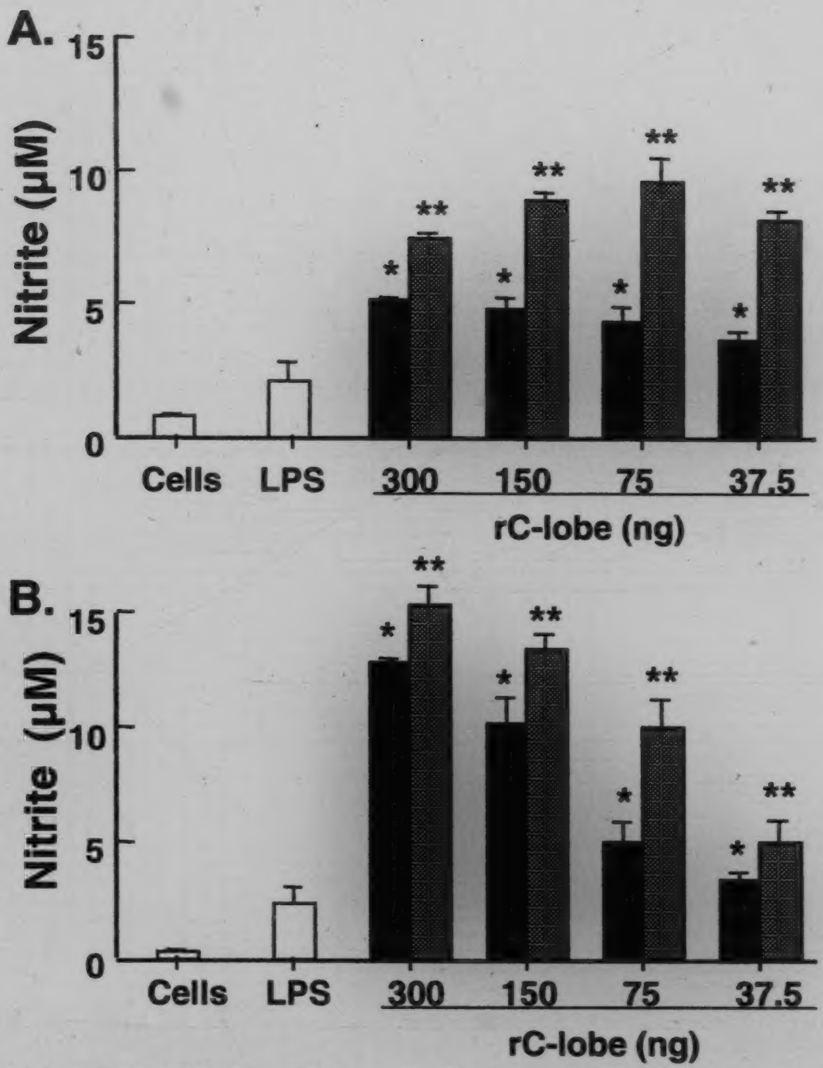

Figure 6.15. Induction of NO production in goldfish macrophages by treatment with recombinant transferrin C-lobe following application to a Detoxi-Gel™ LPS-removal column. One ml sample of purified recombinant protein was applied to a Detoxi-Gel™ column and incubated for 1 h at 4°C. Sample was then eluted from the column with 1xPBS and tested for NO-inducing activity immediately (A) or following an overnight dialysis vs. 4 L of 1xPBS (B). Goldfish macrophages ($5x10^4$ cells/well) were treated with medium (cells), LPS (1 µg/ml) (L), recombinant protein alone (37.5 to 300 ng; black bars), or recombinant protein (37.5 to 300 ng) in conjunction with LPS (1 µg/ml; grey bars) for 72 h at 20°C prior to determination of NO production using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and the results are from a representative experiment of two independent experiments performed. * $P<0.05$ vs. unstimulated cells. ** $P<0.05$ vs. LPS stimulated cells.

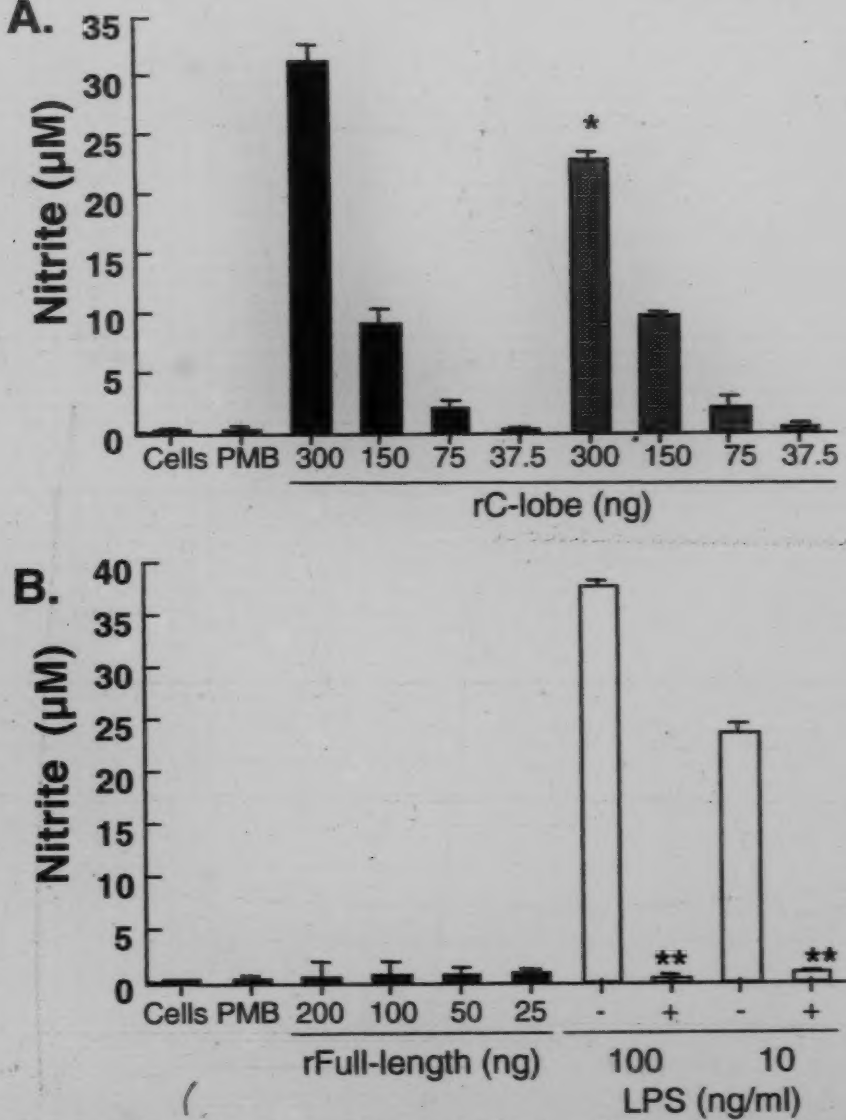

Figure 6.16. Effect of recombinant goldfish transferrin C-lobe on the production of NO in P388D.1 murine macrophage-like cells. P388D.1 cells ($5x10^4$ cells/well) were treated with recombinant goldfish transferrin C-lobe (37.5 to 300 ng) in the absence (black bars) or presence (grey bars) of 10 µg/ml of polymixin B (PMB) (A). Cells were also treated with full-length recombinant goldfish transferrin (25-200 ng) or LPS (100 and 10 ng/ml) in the absence (-) or presence (+) of PMB (B). Cells were incubated for 24 h at 37°C prior to determination of NO production using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and the results are from a representative experiment of two independent experiments performed.
* $P<0.05$ vs. cells treated with 300 ng rC-lobe in the absence of PMB; ** $P<0.05$ vs. cells treated with the same concentration of LPS in the absence of PMB.

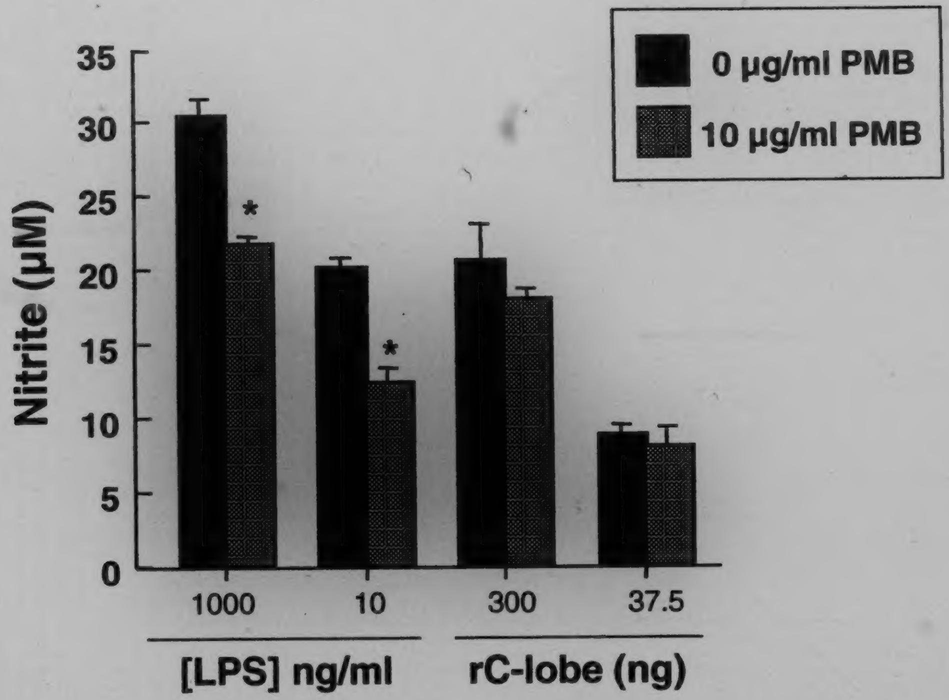

Figure 6.17. Effect of recombinant goldfish transferrin C-lobe on the production of NO in bone marrow-derived murine macrophages. Macrophages (5x10^4 cells/well) were treated with LPS (1000 and 10 ng/ml) or recombinant C-lobe (300 and 37.5 ng) in the absence (black bars) or presence of 10 µg/ml of the LPS-inhibitor polymixin B (PMB). Cells were incubated for 24 h at 37°C prior to determination of NO production using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and the results are from a representative experiment of two independent experiments performed. * $P<0.05$ vs. cells not treated with PMB.

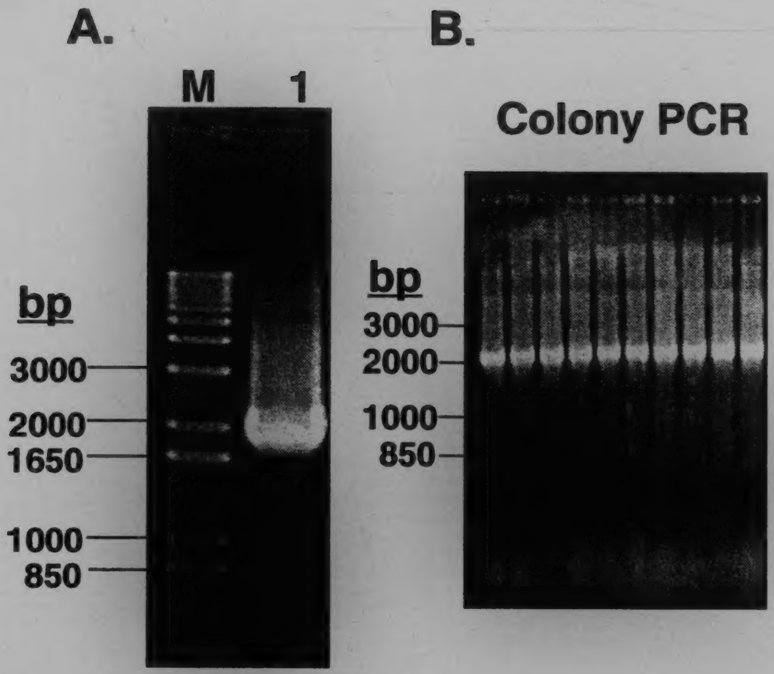

Figure 6.18. RT-PCR and cloning of goldfish transferrin into the eukaryotic expression vector pIB/V5-His TOPO®. RT-PCR was performed using the goldfish transferrin expression primers and goldfish liver cDNA was used as a template (A). Lane M, 1 Kb Plus DNA Ladder (Invitrogen); Lane 1, full-length goldfish transferrin PCR product amplified using eukaryotic expression construct primers. PCR product was cloned into the pIB/V5-His TOPO® expression vector and inserts detected by colony PCR using the vector specific primers OpIE2 (B). Lane M, 1 Kb Plus DNA Ladder (Invitrogen). Shown are ten randomly picked colonies used for detection of cloned inserts. DNA visualized following separation on a 1.2% agarose gel stained with EtBr.

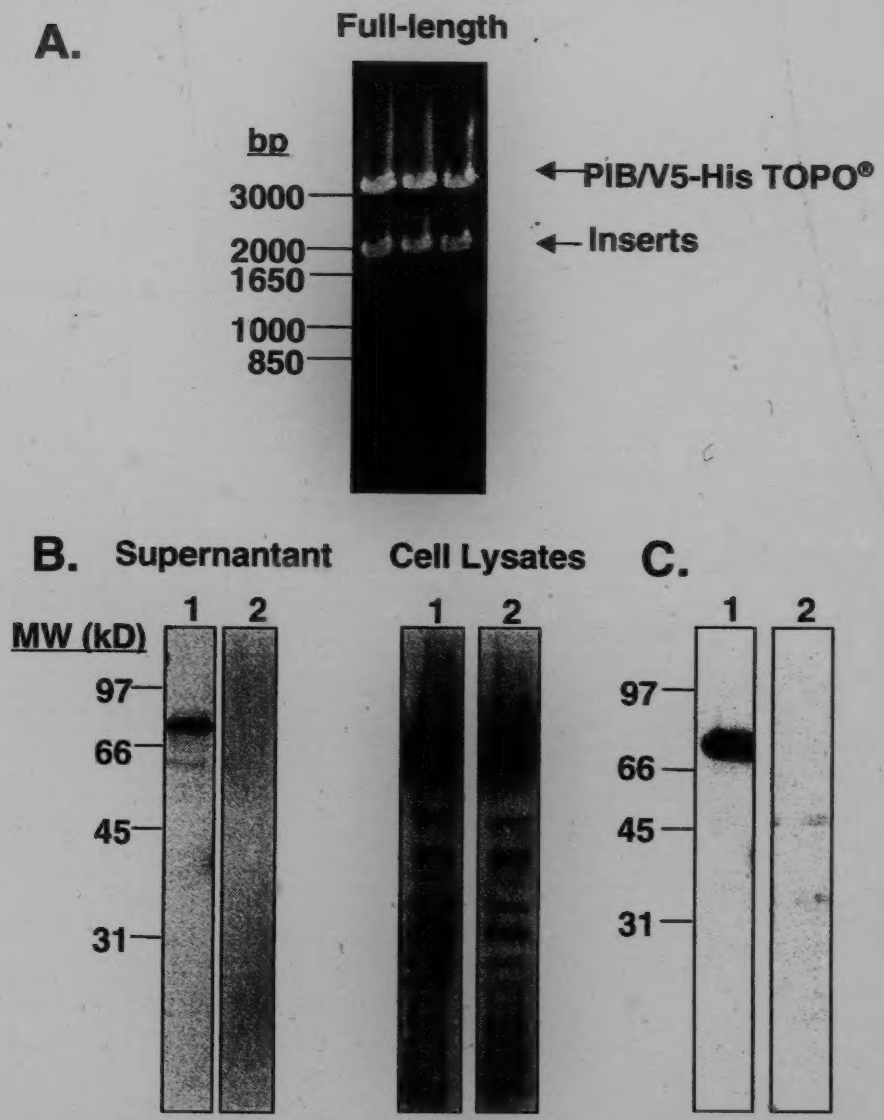

Figure. 6.19. Expression of recombinant goldfish transferrin in the insect cell-line Sf9. Verification of cloned inserts into the pIB/V5-His TOPO® expression vector was performed by a restriction digest with BamH1 and Not 1 followed by visualization on a EtBr stained 1.2% agarose gel (A). Detection of recombinant protein expression was performed by separating cell supernatants or lysates from transiently transfected Sf9 cells by reducing SDS-PAGE, which were then transfered to nitrocellulose membranes and probed with an anti-V5 monoclonal antibody (1:5000) (B); Lane 1 transfected cell; Lane 2, non-transfected control. Supernatants from Blastacidin-selected Sf9 cells were also examined for the presence of recombinant protein expression (C); Lane 1; transfected cells; Lane 2, non-transfected control.

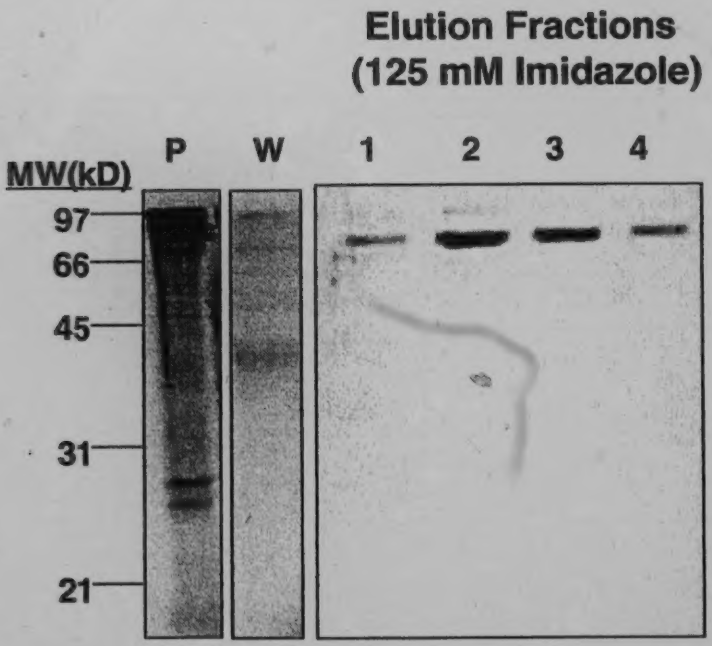

Figure 6.20. Purification of recombinant goldfish transferrin from the supernatants of a polyclonal cell-line derived from Blasticidin-selected Sf9 insect cells. Supernatants were collected, dialysed overnight vs. NiNTA buffer and concentrated prior to application to a 5 ml NiNTA agarose column. Samples were allowed to flow through column by gravity and bound His-tagged recombinant proteins were eluted with 125 mM imidazole. Lane P, pre-NiNTA dialyzed supernatant; Lane W, wash fraction containing NiNTA unbound proteins; Numbered lanes represent imidazole elution fractions for removal of bound recombinants from the column. Shown is a reducing SDS-PAGE of separated samples after staining with Coomassie blue.

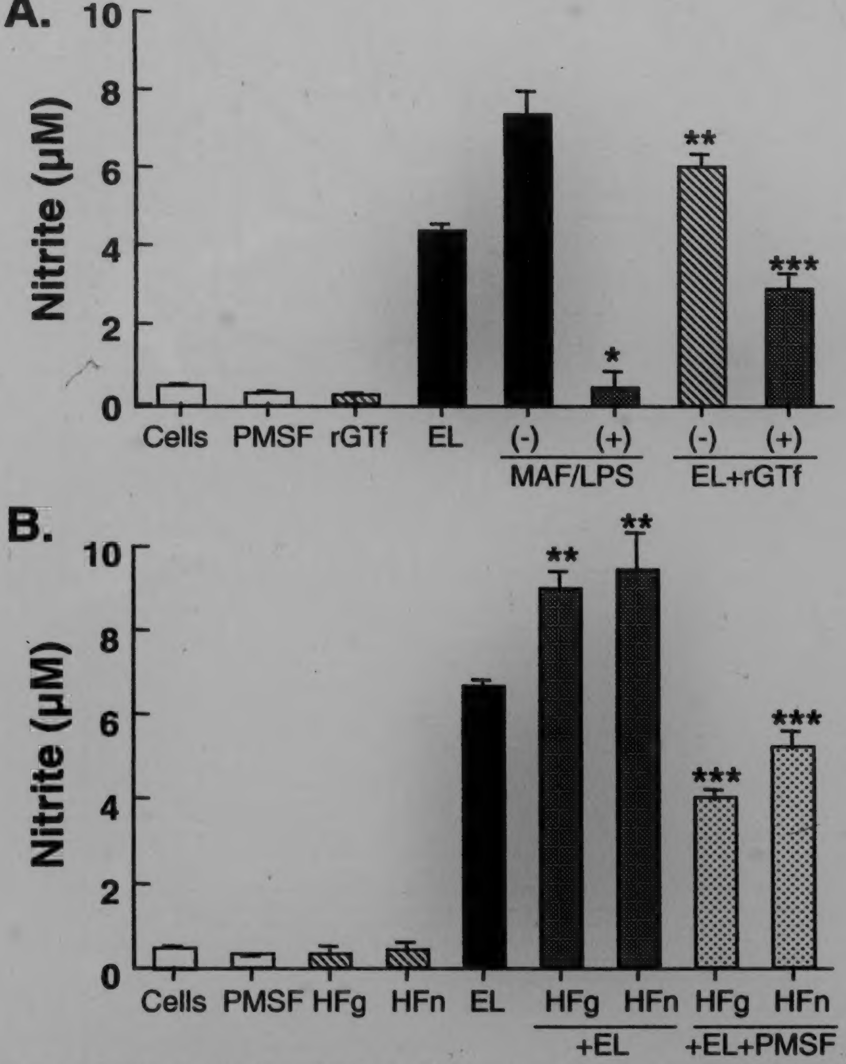

Figure 6.21. Induction of NO production in goldfish macrophages by elastase alone or in combination with host proteins. Goldfish macrophages ($5x10^4$ cells/well) were treated with medium alone (cells), 10 mM of the enzyme inhibitor PMSF, 1 μg of recombinant full-length goldfish transferrin (rGTf), MAF (1:4) and LPS (1 μg/ml), 10 μg of elastase (EL), or 1 μg of rGTf and EL; in the absence (-) and presence (+) of 10 mM PMSF (A). Cells were also treated with 10 μg of human fibrinogen (HFg) or fibronectin (HFn) alone or in combination with 10 μg elastase alone (+EL) or elastase +PMSF (EL+PMSF) (B). Treated goldfish macrophages were incubated for 72 h at 20 °C prior to determination of NO production using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and the results are from a representative experiment of two independent experiments performed. * P<0.05 vs. MAF and LPS activated cells without 10 mM PMSF; ** P<0.05 vs. elastase treated cells; *** P<0.05 vs. EL in combination with rGTf, HFg or HFn without PMSF.

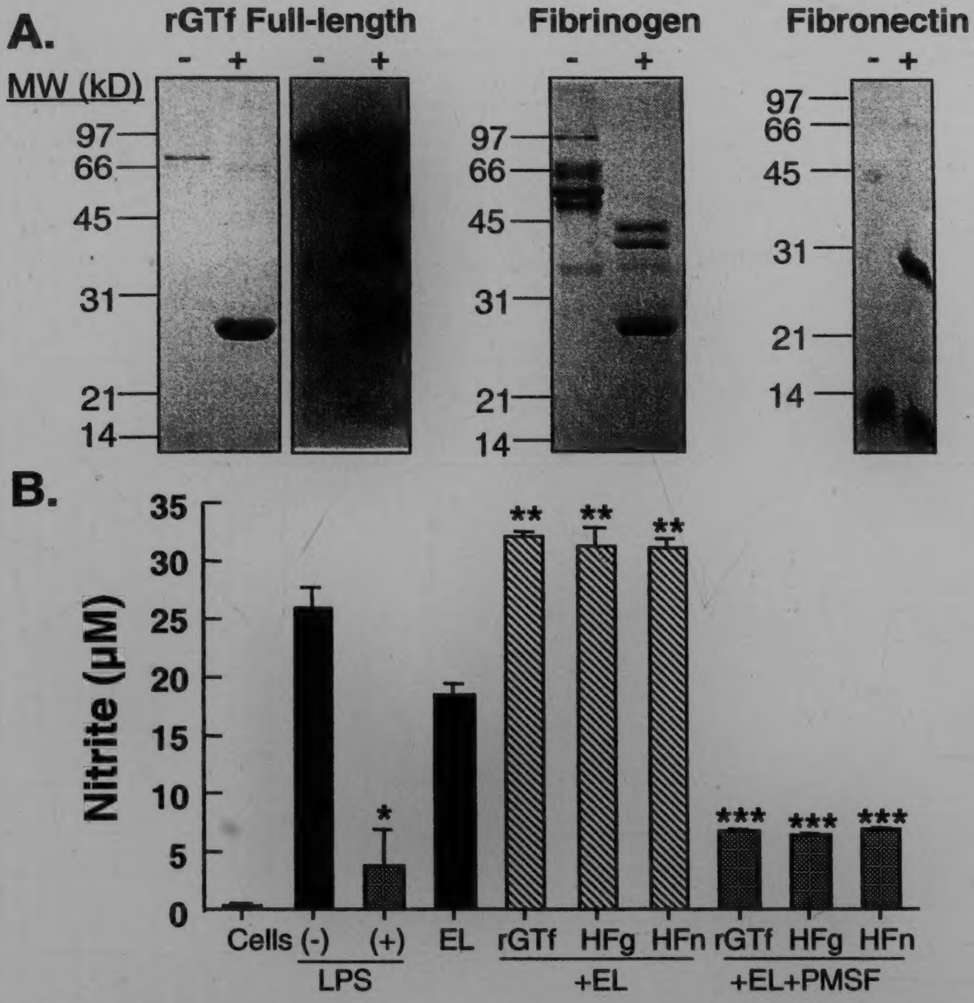

Figure 6.22. Induction of NO production in murine macrophages by elastase alone or in combination with host proteins. Recombinant goldfish transferrin (rGTf), human fibrinogen (HFg), and human fibrinonectin (HFn) were incubated with 1xPBS (-) or with 2 μg of porcine elastase (+) for 2 h prior to separation by SDS-PAGE and staining with Coomassie blue (A). rGTf was also transferred to a nitrocellulose membrane and probed with the polyclonal and carp serum transferrin antibody (9AG7; right panel). P388D.1 murine macrophage-like cells were treated with medium (Cells) or 100 ng LPS alone (-) or with (+) 10 mM of the protease inhibitor PMSF (B). Cells were also treated with elastase alone (5 μg; EL) or with EL in conjunction with rGTf (1 μg), human fibrinogen (HFg; 10 μg), or human fibronectin (HFn; 10 μg) in the absence or presence of PMSF. Cells were incubated for 24 h at 37°C prior to determination of NO production using the Griess reaction. Each bar represents the mean ± SEM of triplicate cultures and the results are from a representative experiment of two independent experiments performed. * $P<0.05$ vs. LPS without 10 mM PMSF; ** $P<0.05$ vs. EL alone; *** $P<0.05$ vs. EL in combination with rGTf, HFg or HFn without PMSF.

CHAPTER 7

IDENTIFICATION OF A TOLL-LIKE RECEPTOR (TLR) GENE THAT IS UP-REGULATED IN ACTIVATED GOLDFISH MACROPHAGES[6]

7.1 Introduction

Innate immune surveillance relies in part on the recognization of conserved molecules unique to some classes of potential pathogens. For example, bacterial lipopolysaccharide (LPS) and lipoteichoic acid (LTA) found on the surfaces of gram negative and gram positive bacteria, respectively, are potent inducers of immune responses. These and other such immunostimulatory compounds are collectively known as pathogen-associated molecular patterns (PAMPs). PAMPs are bound by host pattern recognition receptors (PRR), which may be secreted or expressed on the surface of immune cells. The Toll-like receptors (TLRs) are a family of cell surface proteins that have been shown to provide intracellular signalling following either direct binding of PAMPs or binding of ligand-engaged PRRs (reviewed in Chapter 1). In mammals, TLR signalling can initiate both innate and adaptive immune responses [632].

The Toll receptor was originally identified as a key mediator of development in *Drosophila* [116], and only later was it found to also initiate innate antifungal responses in the fly [9]. Subsequently, a family of Toll receptors have been identified in *Drosophila*, as well as in mammal [695], and birds [696]. Like *Drosophila* Toll, these vertebrate Toll-like receptors initiate second-messenger pathways that regulate the expression of genes that are required for a protective immune response. For example, activation through TLRs has been shown to induce potent inflammatory responses, which include the production of reactive oxygen and nitrogen intermediates, secretion of chemokines and cytokines, and cellular differentiation, many of which are regulated by an NF-κβ-mediated signaling cascade [6]. Thus, these receptors represent an evolutionarily conserved system of innate immunity.

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[6] A version of this chapter has been published: Stafford *et al.*, 2003. *Dev. Comp. Immunol.*, 27: 685-698.

Toll-like receptors are characterized by an N-terminal extracellular domain containing several leucine-rich repeats (LRRs), and an intracellular C-terminal Toll/Interleukin-1 response (TIR) domain, named for its homology to the signaling domain of the IL-1 receptor. The TIR domain interacts with a heterotrimeric complex of death-domain containing adapter proteins [117], ultimately leading to the activation of the transcription factor NF-κβ or its homologues, Dif or Relish, in *Drosophila*. Leucine-rich repeats are a commonly found structural motif that has been widely implicated in mediating protein-protein interactions [697]. LRR domains have conformational flexibility, and feature solvent-exposed β-sheets that can participate in strong hydrophobic interactions [698]. Thus, the LRR domains of TLRs may play a key role in the recognition and binding of ligand engaged PRRs or various PAMPs found on a variety of different classes of pathogens. For example, TLR-4 forms a complex with LPS and another cell surface PRR known as CD14 in combination with a linker protein (MD2), to form the functional signal transduction receptor for LPS [129-131]. In addition to LPS and other foreign molecules, it has recently been shown that TLR-4 can also bind a diverse array of endogenous proteins. For example, several endogenous ligands have been implicated as potential activators of TLR-4 and include fibrinogen [10], fibronectin [11], surfactant protein-A [152], heat-shock proteins [153-154], hyaluronan [155], and heparan sulfate [156].

Until recently there was no conlusive evidence that TLRs existed in fish, as there were no published reports on the identification of an expressed teleost TLR homologue. Others have implicated that teleost TLRs may be present and important for the generation of immune responses in fish. For example, several EST projects have identified sequence fragments with significant homology to regions of mammalian TLRs (Zebrafish: GenBanks accessions BM185313, BG304206, BF158452; Japanese flounder AB076709, AU091257; Rainbow trout: AF281346); however, none of these sequences contained both the TIR and LRR domains, which are hallmarks of TLRs. The recently released *F. ruburipes* genome database [158] and analysis for fish homogues of the TLR family revealed that the Toll family is shared by fish and humans. The predicted Fugu TLR-2, -3, -5, -7, -8, and –9 corresponded structurally to the respective mammalian TLRs and one Fugu TLR showed equal amino acid homologies to human TLR-1, -6, and –10 [159]. Interestingly, two of the Fugu genes were found to be unique to fish and were named TLR-21 and –22. The pufferfish genome provides evidence of several possible TLR genes, and the results of this chapter verified that at least one of these receptors was expressed on goldfish macrophages.

In this chapter, I report that an EST screen of an activated goldfish macrophage subtractive library generated a transcript, which was identified as a putative teleost TLR. I then obtained the full-length goldfish TLR cDNA using 5'- and 3'-RACE, which was subsequently sequenced. The full-length transcript encoded a type I transmembrane protein with an extracellular domain containing LRR and a cytoplasmic tail encoding a TIR domain. This goldfish TLR does not have distinguishable sequence homology with any single mammalian TLR that has been described but contains both a LRR and a TIR domain, which are hallmarks of the Toll and TLR family members. Finally, the results of this chapter demonstrated that the goldfish macrophage TLR was constitutively expressed in goldfish spleen and kidney but not heart and liver and that induction of TLR expression was achieved following treatments with both endogenous and exogenous factors.

7.2 Results

7.2.1 Analysis of goldfish TLR cDNA and predicted polypeptide

A goldfish TLR was originally identified as a 412 bp EST from an LPS/MAF stimulated goldfish macrophage subtracted library. Complete sequence of the goldfish TLR was obtained by 5'- and 3'-RACE using primers based on the EST sequence (Fig. 7.1 and Fig. 7.2, respectively). The nucleotide sequence of the full-length goldfish TLR is 3553 bp and was submitted to GenBank (Accession# AY162178) and contained an open reading frame of 2838 nucleotides, encoding a putative protein of 945 amino acids (Fig. 7.3). The predicted start codon was the first in-frame ATG following a stop codon and was 162 bp at the start of the transcript.

Database sequence homology searches of the predicted goldfish TLR amino acid sequence was performed using NCBI BLASTP and revealed that the predicted protein had significant homology to a variety of TLRs previously submitted to the database. The highest BLASTP identity match for the full-length goldfish TLR was 30% amino acid identity with the murine TLR7 precursor (GenBank Accession#

XP_142018). Analysis of the predicted goldfish TLR revealed the presence of several distinguishing features indicating that this protein was indeed 'Toll-like', including LRR domains found between amino acids 88-682, transmembrane α-helices between amino acids 735-780, and a TIR domain located between amino acids 792-935. This 945 amino acid protein had a predicted MW of 109 kDa, and contained a signal peptide in the first 32 amino acids.

7.2.2 Alignment and phylogenetic analysis

Alignments of the amino acid sequence of goldfish TLR with a selection of a few of the highest homology matches from the BLASTP results was performed (Fig. 7.4) using CLUSTAL W 1.81, as described in Materials and Methods (Chapter 2). When the full-length amino acid sequences were compared (i.e. both LRR & TIR domains), goldfish TLR demonstrated 26.7%, 27.5%, and 24.7% identity with mouse TLR-8, mouse TLR-7, and human TLR-3, respectively. The regions of highest amino acid identity were evident within the TIR domains of the different TLRs (Fig. 7.4). To determine whether goldfish TLR had clear homology with any specific human TLR, I analyzed amino acid identity of goldfish TLR versus the 10 known human TLRs (Table 7.1). The LRR and TIR domains did not exhibit the same relative homologies to any given human TLR though in all cases the homologies among TIR domains was greatest (Table 7.1). I then developed a phylogenetic tree, to assess relatedness of goldfish TLR, based just on its TIR domain. The TIR domain of goldfish TLR did not cluster with any of the tetrapod TIR domains (Fig. 7.5), however, it did cluster with a predicted TLR from the *Fugu ruburipes* genome database [158]. Inclusion of a recently identified *Salmo salar* interleukin-1 receptor sequence (Genbank Accession# AJ293335), in the phylogenetic tree analysis did not have a significant effect on the clustering of the goldfish TLR and was not included in the phylogenetic tree.

7.2.3 RT-PCR analysis of TLR mRNA expression

RT-PCR analyses of goldfish tissues from apparently healthy goldfish indicated that spleen and kidney but not liver or heart expressed constitutive levels of the TLR message (Fig. 7.6A). Spleen and kidney both contain sizable macrophage populations. However, since levels of TLR mRNA were barely detectable in non-stimulated macrophages even by RT-PCR, I reasoned that goldfish TLR expression levels in tissues would not be detectable by Northern blot analysis.

Constitutive low-level TLR expression was observed by RT-PCR in unstimulated goldfish macrophages though levels appeared to increase within 3 to 6 h following LPS and MAF stimulation (Fig. 7.6B). At 24 h post-stimulation, a decreased level of TLR expression was seen. The observed induction of iNOS mRNA was consistent with the cells having been activated. β-actin mRNA expression was monitored to demonstrate an intact cDNA template, the absence of genomic DNA contamination and equal gel loading.

7.2.4 Examination of TLR mRNA expression by goldfish macrophages using northern blot analysis

As shown in Figure 7.7, low but detectable levels of TLR expression were observed in unstimulated goldfish macrophages (control). Notably, two bands on the

Northern blots were detected with sizes of ~5.3 and ~3.5 kb. The smaller band corresponded to the size of the original full-length TLR transcript identified in this study, while the larger band (~5.3 kb), which was present in almost identical quantities for all treatment groups, may represent an alternately processed form of this receptors pre-mRNA. The probe used for the Northern blot encompassed a 2586 bp region of the goldfish TLR from bases 305 to 2890. An independent northern analysis using a smaller probe (390 bp) spanning bases 2501 to 2890 of the gene showed an identical pattern of bands (data not shown). Several independent PCRs performed using goldfish genomic DNA as template generated a single 2586 bp band which suggests that no introns were present between bases 305 and 2890 of this sequence. RT-PCR of cultured macrophages and various tissues also generated a single 2586 bp band. Therefore, any alternate pre-mRNA processing likely occured either 5' of base 305 or 3' of base 2890 and the two bands observed following 3'-RACE (Fig. 7.2A) suggested that the alternate processing occured at the 3' end of the gene.

Northern blot analyses corroborated the RT-PCR studies of TLR mRNA expression in MAF/LPS stimulated macrophages (Fig. 7.7). As indicated by RT-PCR analysis, goldfish TLR expression was up-regulated at the 3 and 6 h time-points following stimulation with MAF and LPS (Fig. 7.6B). To further delineate the kinetics of expression of the goldfish macrophage TLR, Northern blot experiments were performed. Treatment with LPS alone caused an 8-fold up-regulation of TLR expression after 3 h, which continued to increase at least 6 h post-stimulation (Fig. 7.7). After 24 h, TLR expression had measurably declined in macrophages exposed to LPS, though not down to control levels (relative expression level of control was 1.0). Exposure to MAF supernatants did seem to have a slight inductive effect on TLR expression after 3 and 6 h post-stimulation (1.5- and 3-fold, respectively), however, the expression levels of the TLR gene at 24 h following MAF treatment were lower than the untreated control. Simultaneous exposure of macrophages to MAF and LPS induced a similar pattern of increased TLR expression to that by macrophages stimulated with LPS alone at all time points measured. Stimulation with heat-killed *A. salmonicida* or live *M. chelonei* also caused up-regulation of TLR at 3, 6 and 24 h post-stimulation, though with different kinetics from that of LPS or each other. Declines in TLR expression were also seen 24 h after stimulation with whole bacteria.

7.2.5 Southern blot analysis of the goldfish TLR

To determine whether the two bands on the Northern blot were the products of two highly homologous TLR genes, I performed a Southern blot of goldfish genomic DNA using the same probe as used for the Northern blot (Fig. 7.8). Digests of the DNA with two of the enzymes (Pst I and Hind III) predicted to only cut outside of the TLR gene generated a single major band, which was indicative of a single gene with conserved flanking restriction sites. Both the Bam HI and Sph I digests had two major hybridizing bands. Bam HI was not predicted to cut within the goldfish TLR so the bands may have resulted from restriction fragment length polymorphisms. Sph I was predicted to cut within TLR and should have generated at least two hybridizing bands. Weakly hybridizing bands were also present in all lanes and may represent other TLR family members.

7.3 Discussion

The results presented in this chapter provided evidence for the identification of one of the first full-length teleost TLR cDNAs. Analysis of the sequence revealed a large open reading frame with an amino acid translation of 945 residues. Protein motif analyses indicated the presence of two distinct features that are hallmarks of TLRs. The cytoplasmic tail of the predicted protein had high homology (> 40% amino acid identity) with the TIR domains of other reported TLRs. Straddling the other side of the putative transmembrane domain was an area rich in LRR motifs. Identification of these conserved domains in combination with sequence alignments and gene expression patterns, clearly indicated that this was a TLR homologue in teleost. The existence of two bands on the northern blot suggested the presence of a longer alternately processed form of this receptor. This would not be without precedent as human TLRs -4, -8 and -9 have multiple splice variants entered into the Genbank database (Genbank accessions: TLR4-A: NM_138554; TLR4-B: NM_138556; TLR4-C: NM_003266; TLR4-D: NM_138557; TLR8-1: NM_016610; TLR8-2: NM_138636; TLR9-A: NM_017442; TLR9-B: NM_138688). To my knowledge the expression profiles of these alternatively splice variants has not been reported in the literature. PCR using the RT-PCR primers and genomic DNA as template suggested the absence of an intron between bases 305 and 2890 of this goldfish TLR. Therefore any alternate splice site would likely exist outside of these boundaries. One possibility was that these pseudo-tetraploid fish have two TLR genes with regions of high homology that give rise to different size transcripts. However, Southern blot analysis indicated that only 1 copy of this gene was present within the goldfish haploid genome. I recently isolated and sequenced a second rare 3'-RACE product (Fig. 7.2A; ~4.5 Kb fragment). This second RACE product overlaped perfectly with the 3.5 kb transcript in over 600 nucleotides of coding sequence and all of the 3' untranslated portion of the 3.5 kb transcript (data not shown). This second, larger, 3'-RACE product had an additional 1.3 kb of 3' untranslated region terminating in a poly(A) tail, and may account for the larger band observed in the Northern blots. The Genbank submission for the goldfish TLR (AY162178) has been amended to include this additional 3' untranslated region.

To date, there has been relatively little evidence for the identification of an expressed teleost TLR homologue, although the results of this chapter confirmed that they do indeed exist. Others have implicated that teleost TLRs may be present and important to the generation of immune responses in fish. For example, several EST projects have identified sequence fragments with homology to regions of mammalian TLRs (Zebrafish: Genbank accessions BM185313, BG304206, BF158452; Japanese Flounder AB076709, AU091257; Rainbow trout: AF281346); however none of these sequences contained both identifiable TIR and LRR domains and as such cannot yet be classified as *bona fide* TLRs. The recently released *Fugu ruburipes* genome database [158] includes several predicted TLR sequences. However, until recently, there was no information indicating whether these predicted TLR genes were expressed [159].

Expression profiles for goldfish TLR determined by Northern blot analysis indicated that there was an up-regulation of goldfish macrophage TLR expression in response to various stimuli. Exposure of goldfish macrophages to LPS, heat-killed *A. salmonicida*, or live *M. chelonei* each caused a significant induction of the TLR gene when compared to untreated cells. However variations in activation kinetics between the different treatments were also evident. Goldfish macrophages exposed to heat-killed *A. salmonicida* exhibited higher levels of TLR expression after 3 and 6 h compared with macrophages exposed to live *M. chelonei* (4- and 8-fold vs. 3- and 6-fold, respectively). However, after 24 h, macrophages exposed to *M. chelonei* expressed slightly higher levels of TLR. I have not yet established whether these variations were dose-related, or may represent responses to different stimulatory PAMPs present in the treatments. For example, the progression of the immune response to LPS may differ from the response to mycobacterial lipopeptides. As well, while the *A. salmonicida* treatment involved heat-killed bacteria, the *M. chelonei* treatment did not, and the presence of a live infection may have influenced the expression patterns observed. I have also not yet resolved how to limit growth of *A. salmonicida* such that it does not overwhelm macrophage cultures when administered live. In mammals, there was precedence for the up-regulation and differential expression of various TLRs in response to specific combinations of cytokines or bacterial components. For example, in murine macrophages TLR-2 but not TLR-4 were induced in response to bacterial infections and may accelerate the innate immune response against pathogens [714], Also, TLR-9 was up-regulated following LPS stimulation of mouse macrophages [715]. Both TLR-2 and TLR-4 were also up-regulated during intestinal inflammation in humans [699], and there was an alteration in the intestinal epithelial cell expression of TLR-3 and TLR-4 in inflammatory bowel disease [700]. The down-regulation of TLR expression has also been reported. In response to IL-4, expression of the TLR-4 gene was down-regulated by human peripheral monocytes but not B-cells and this phenomenon was a proposed mechanism for directing the induction of a humoral immune response [701]. In addition, LPS-tolerance *in vivo* was shown to result from the down-regulation of TLR-4 surface expression in mouse peritoneal macrophages [702], which may be due to a decrease in the production of inflammatory cytokines [703].

Toll-like receptors have been identified in a wide range of organisms including *D. melanogaster* and humans and play a central role in the recognition of pathogens and initiation of immune responses. The recently published pufferfish genome provided evidence of several possible TLR genes, and my results verified that at least one of these TLRs was expressed on goldfish macrophages. This goldfish TLR does not have distinguishable sequence homology with any disitinct mammalian TLR that have been previously described, nor can we conclude or speculate on the potential ligands recognized by this receptor. Further studies are required to elucidate the specific functional characteristics of this goldfish macrophage TLR. Since goldfish macrophages recognize and respond to a variety of pathogenic challenges, it will be interesting to examine what specific role different TLRs play in the generation of these responses. In addition, the specificity of this receptor for pathogen and/or endogenously derived signals will be necessary for further elucidating its role in innate immune responses of goldfish.

Table 7.1 Amino acid identity comparisons of goldfish macrophage TLR domains with various human TLR domains

Goldfish TLR domains	Human (TLRs) and % Identity with Goldfish TLR									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
TIR[1]	41.0	40.1	36.1	35.9	32.9	40.0	36.4	37.8	29.4	38.2
LRR[2]	13.5	17.5	19.9	18.0	19.3	13.6	23.4	24.2	22.8	14.6
LRR & TIR[3]	18.3	21.2	24.7	22.5	22.8	18.3	25.4	26.0	23.9	19.0

[1] Toll/interleukin-1 receptor domain.
[2] Leucine rich repeat domain.
[3] Full-length TLR.

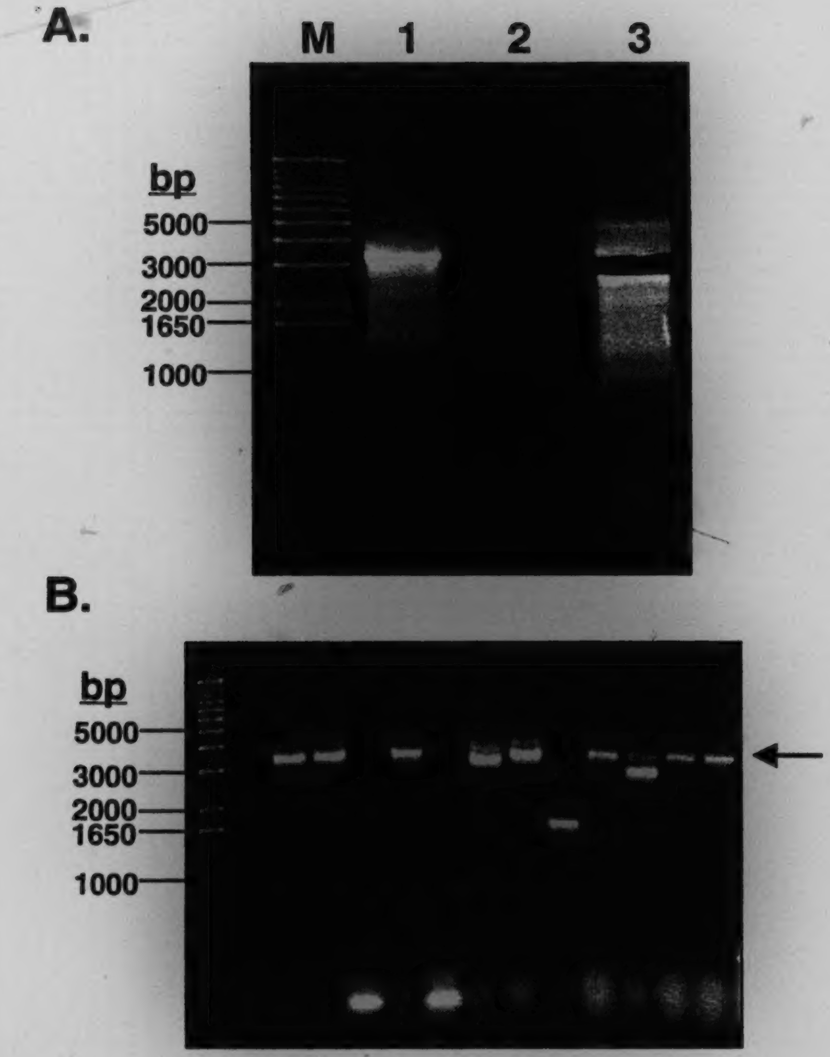

Figure 7.1. 5'-RACE and cloning of a goldfish TLR. 5'-RACE was performed using an antisense gene-specific primer (Table 1.1; Chapter 1) based on the original expressed sequence tag (EST) identified from activated goldfish macrophage cDNA. Shown in (A) is the 5' RACE product, which was ~3000 bp. Lane M, 1 Kb Plus DNA Ladder; Lane 1, 5' RACE product; Lane 2, 5' RACE performed without any template; Lane 3; excised 5' RACE product used for cloning and subsequent sequencing. Excised 5' RACE product was sub-cloned into the pCR2.1-TOPO® vector and colony PCR performed to identify clones with appropriate sized inserts (indicated by arrow), which were then identified following DNA sequencing (B). PCR products were separated on a 0.8% agarose gel and visualized following staining with EtBr.

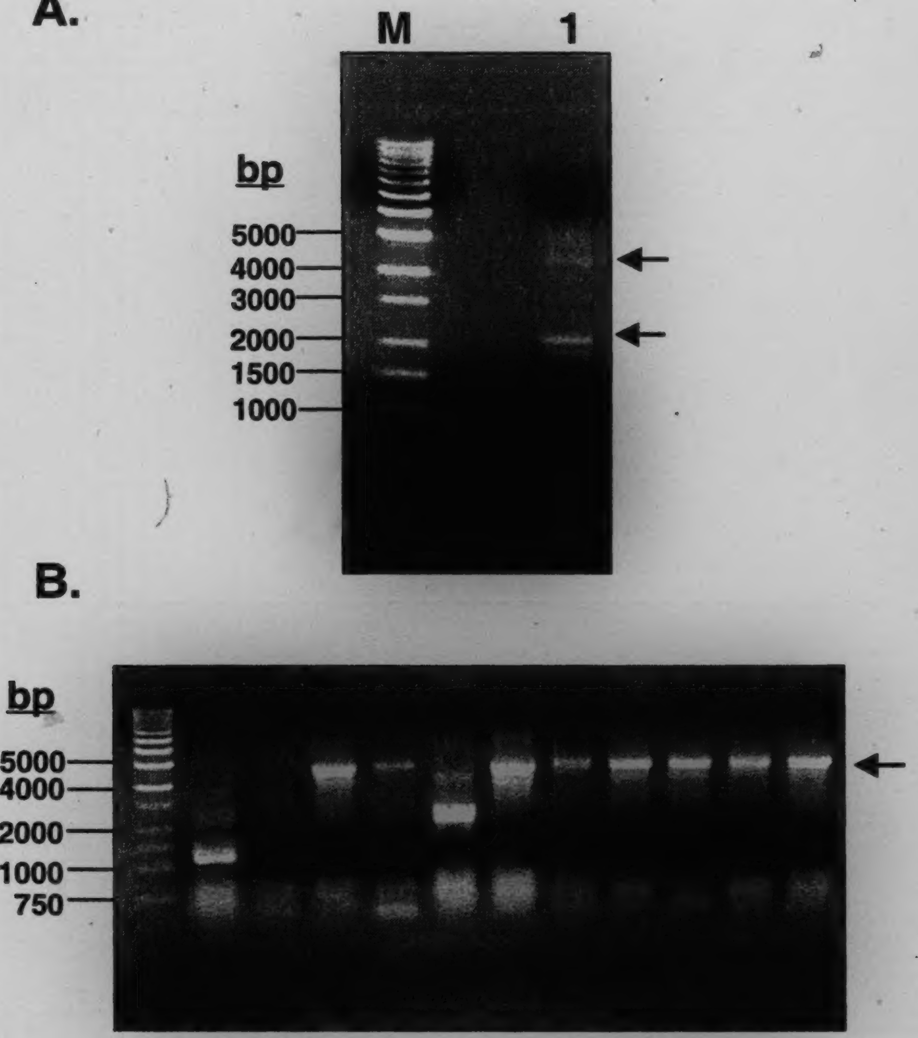

Figure 7.2. 3'-RACE and cloning of a goldfish macrophage TLR. 3'-RACE was performed using a sense gene-specific primer (Table 1.1; Chapter 1). Shown in (A) is the two products identified following 3' RACE, which were ~4500 bp and ~2000 bp (indicated by arrows). Lane M, 1 Kb Plus DNA Ladder; Lane 1, 3' RACE products. Both the 3' RACE products were excised and sub-cloned into the pCR2.1-TOPO® vector and colony PCR performed to identify clones with appropriate sized inserts (indicated by arrow), which were then identified following DNA sequencing. Shown is the colony PCR for the ~4500 bp fragment, and the cloned insert is indicated by an arrow (B). PCR products were separated on a 0.8% agarose gel and visualized following staining with EtBr.

A.

aaagaagtgtgtgtttgtcagcgagaaatgcaaagtcaaaatcctgagaggctgggcaaagggcacaaaataaattaatttaaaagaagaaaaaga
agaaaacttgtttaggtgaagacagtggattcccattttgacgtgtaaaaagaatcaagaaaaacatg

→

MGTLKQITLYIALFSFISPSSAFSLNNCTISTPLKDIQLKVLCYKMGFFRIPSIPGNTHILDISFNA
FAQIQIGDFNHLSNLQDLNISHNKISQIQEGAFDSLSNLYSLNLASNRLQAISRGMLHGLTNLL
VLRLDNNYIKDIEETAFSTLQNLKVLNLTKNQLHYVEKIKPVLASPHLEELYIGSNHLNAFKSYE
MSTNPLALKILDFSNNPFATFQLTDNIFQFLNHLDLSYCGQNGTMTWNVTEKTFLASVKSLYF
MDVHMSVQNAANVLQSFQNLLNKIRLNGNAELNRTDLLLNACSPVLQVLRLKTNKIKHLTEH
MFDLCSNLTEIDLGDNEISNIPPGMFSGFTQLKTLRLQINKFTRITNFFQNLFTLEFIDLSRNSIK
TLNCDVFANLTQLKMLYLYSNKISIIESCSFKDLKSLEVLRLGANNLLRIGDAFRNGPQSLLEL
QLSFNKLSKIEKYTFRNMSQLKYLHLQDNQISEIESYAFGGLKNLSSLFLSSNKISAKTLTKHQ
DVFSGMPNLENLGLDANIILFSDDQLKFPPFKDLKHLTVLSLHSQRRGIGQIPSNLLQGLSSLE
MFYVGNTNLAHLNPDTFKFSPQLWFLDLSKNALSEDHSIPAELFHPISRLTKLLISRTQLRSLN
FLLNANLSRLSTLKATGNEIDLINKTLIQSLPRLEVLDLQKNTFTCDCDNEFFIDWAKNSNSTQ
VVYLNKYMCSYPSSLRGMSLSAFNTE**SCTLNIDFICFLCSSIVVFLTLLSSFVWHFLRFQVVY**
AYYLFLAFLYDNKKKQSGSTFQYDAFISYNAQDEPWVMEELIPKLEGEQSWRLCLHHRDFE
PGRPIIDNIVDGIYSSRKTICLITNNYLKSNWCSSEVQVASFRLFDEQKDVLILVFLEDIPTHQL
SPYHRMRKLVKKRTYLRWPKPGEDTKIFWQKLKLALEAKEGHKSDPLLIP

*

tgataatatcagaactatatggtttataatgctaaatgtgatttcacaacatctcaagagacccacagtccaatttgatcaactgttaaatgtgttggaaaat
tagttgcactatataaattagttgttgtcatttttaaatatttttatatgtgaagttgacttttatacattttatttacaaatctctgcactgtgtgaatgaacgcaa
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aaaaaaaaaaaaaaaaaa

B.

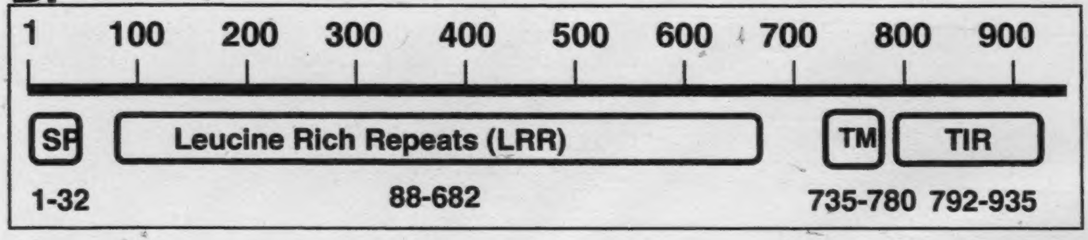

Figure 7.3. Sequence of goldfish macrophage TLR (GenBank Accession# AY162178) (A). The 5' and 3' unstranslated regions of the gene are depicted as nucleotides and the predicted open reading frame shown as amino acids. The putative start codon (ATG) is indicated with an arrow and the stop codon (TGA) indicated by and asterisk (*). Signal peptide (SP; amino acids 1-32) is underlined. The individual leucines are underlined throughout the sequence determined as the leucine-rich region (LRR; amino acids 88-682). The transmembrane domain (TM; amino acids 735-780) is represented as bold letters, and the predicted Toll/IL-1 receptor domain (TIR; amino acids 792-935) has a double underline. A schematic summary of the different regions of the putative goldfish TLR is shown (B).

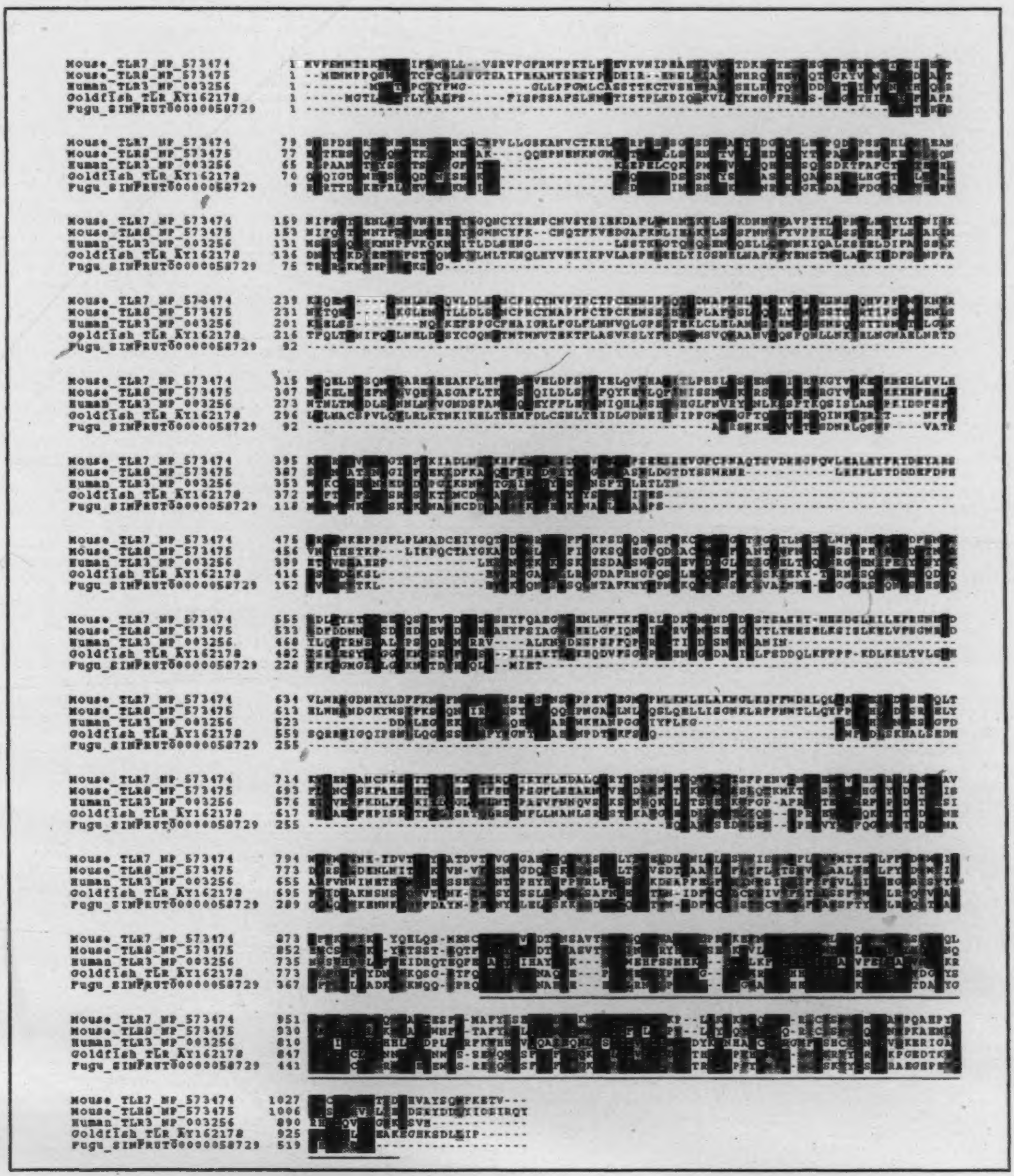

Figure 7.4. Amino acid sequence alignments of the goldfish macrophage TLR protein with fugu, human, and mouse TLR proteins. The goldfish TLR sequence was submitted to GenBank (Accession# AY162178) and aligned with the predicted Fugu TLR (http://www.fugubase.org; SINFRUT00000058729), mouse TLR-7 (NP_573574), mouse TLR-8 (NP_573475), and human TLR3 (NP_003256) using the CLUSTAL W 1.8 program. Dashes indicate gaps, identities are shown as black boxes and shaded boxes represent similar amino acids. The predicted TIR domain is underlined.

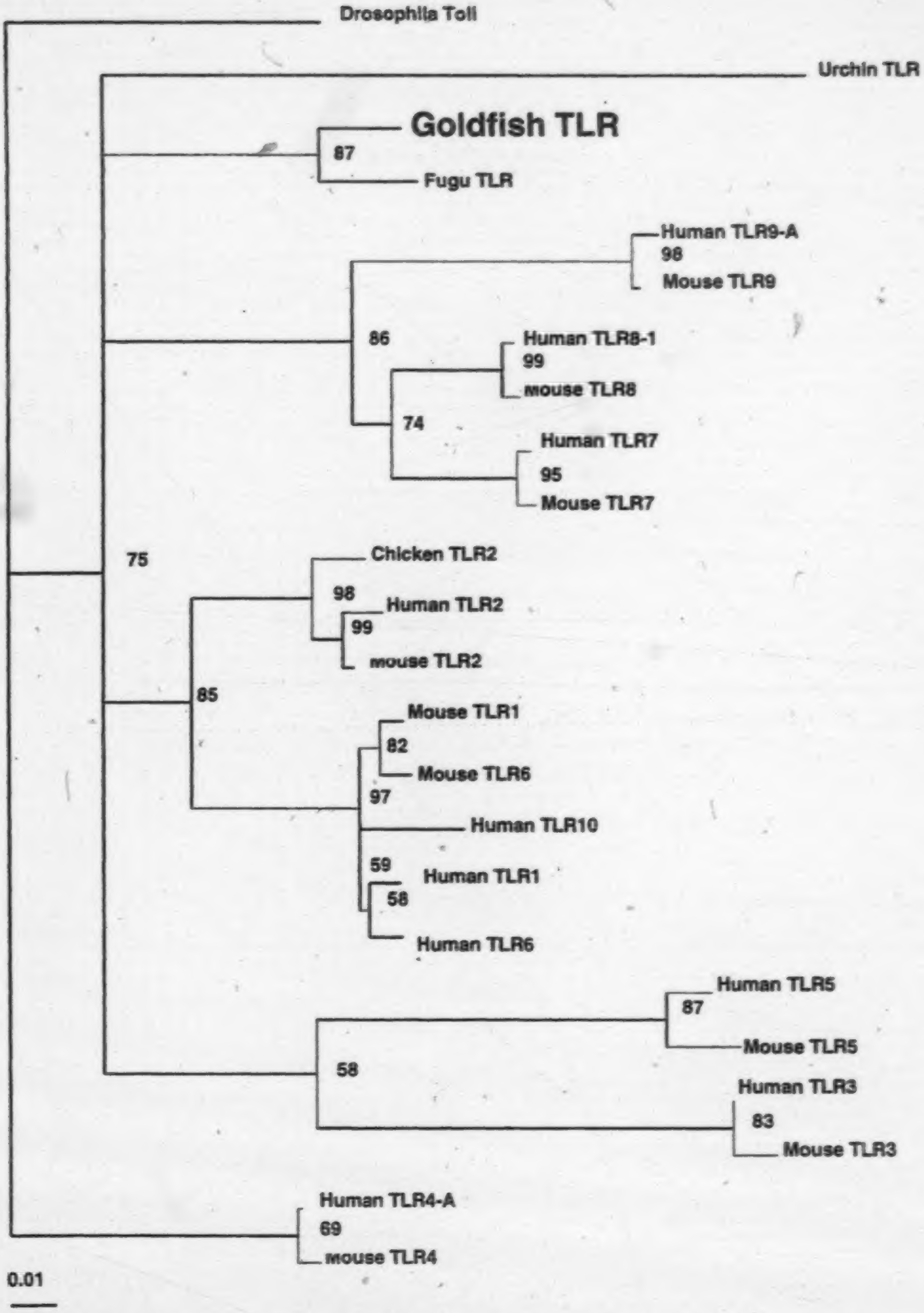

Figure 7.5. Generation of a phylogenetic tree of the TIR domain of various TLR protein sequences using the TREE-PUZZLE program, which constructs phylogenetic trees based on the maximal likelihood using a quartet puzzling algorithm. The TIR domains of the sequences were defined by NCBI conserved domain search. Alignment gaps were included in the analysis.

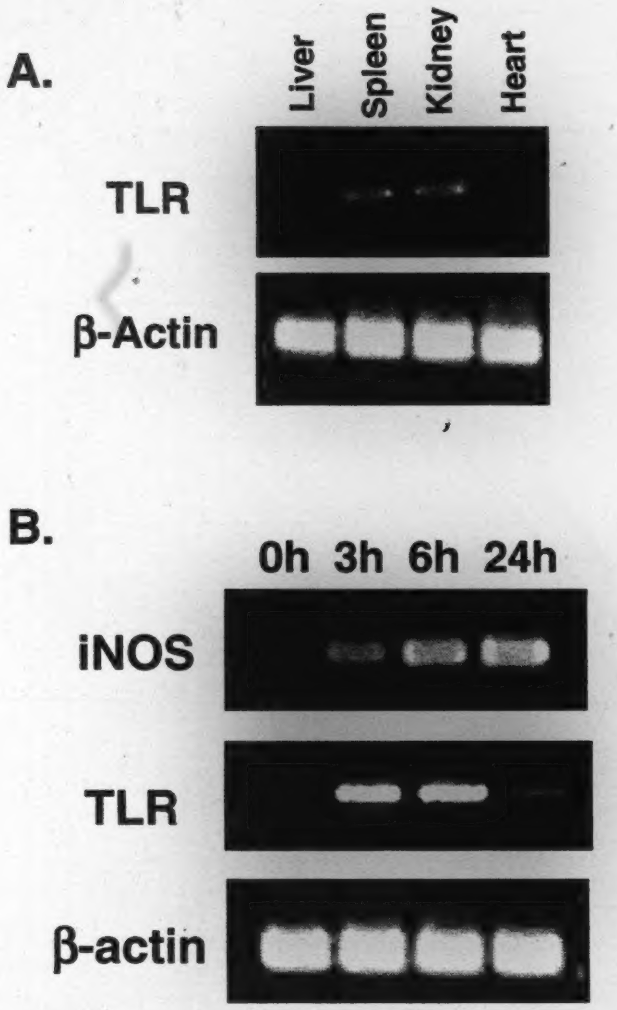

Figure 7.6. RT-PCR expression analysis of goldfish macrophage TLR. Goldfish tissue total RNA was isolated from various tissues and RT-PCR was performed as indicated in the Materials and Methods section. Goldfish TLR gene transcripts were identified in spleen and kidney, but not in liver or heart tissues (A). β-actin expression was also analyzed to confirm the presence of an intact cDNA template. Following stimulation with MAF and LPS, goldfish macrophages TLR expression was also examined after 3, 6, and 24 h post-stimulation (B). Untreated (0 h) cells were included as a control and iNOS expression was included to confirm activation of the macrophages and β-actin expression was also analyzed to confirm the presence of an intact cDNA template.

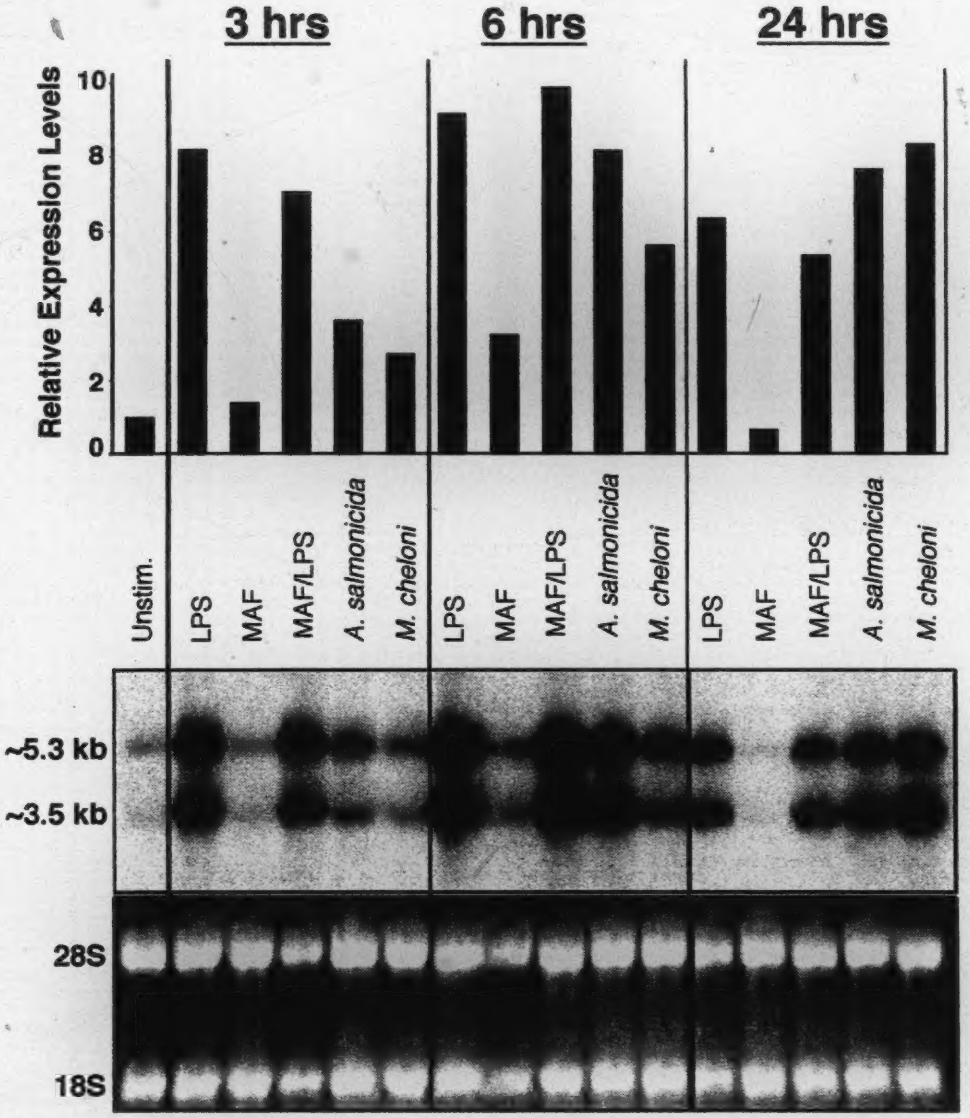

Figure 7.7. Northern blot analysis of goldfish macrophage TLR expression following stimulation with bacterial LPS, MAF, MAF and LPS, heat-killed *Aeromonas salmonicida*, or live *Mycobacterium chelonei*. TLR specific bands from the northern blot (center panel) were measured by densitometry and are provided in the histogram as values relative to the band intensity for unstimulated macrophages. Shown below is the original agarose gel stained with ethidium bromide, which demonstrates the relative amounts of RNA loaded in each lane. Bands of 28S and 18S RNA are indicated.

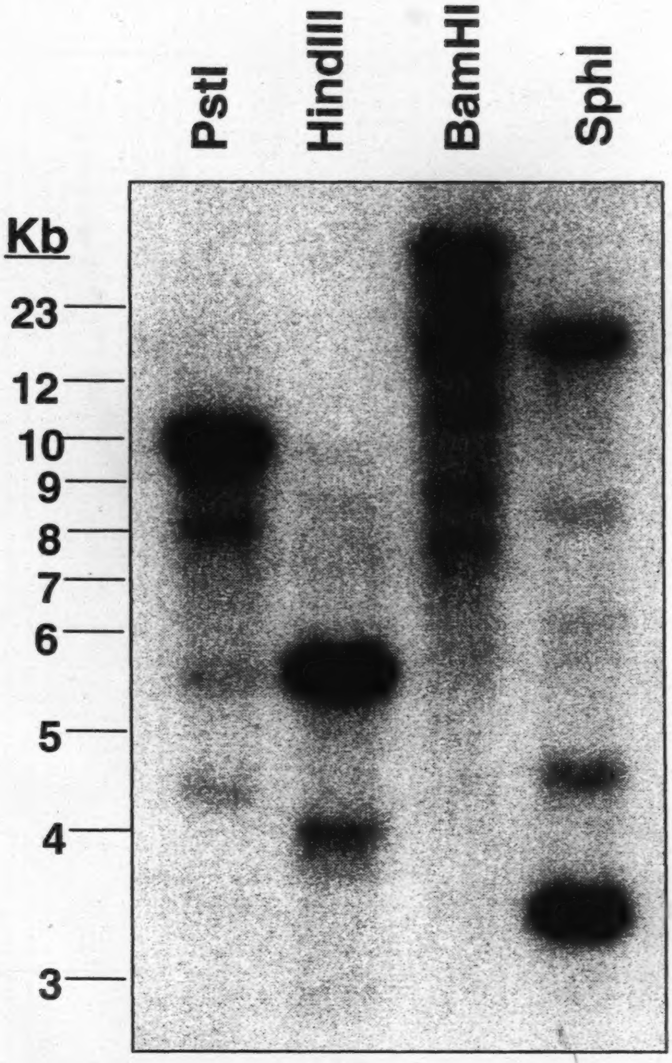

Figure 7.8. Southern blot analysis of goldfish macrophage TLR. Restriction digests were carried out using Pst I, Hind III, Bam HI or Sph I. Only Sph I is predicted to cut within the TLR gene. The probe used for hybridization was identical to that used for Northern analysis. Each lane contains 15 µg of digested goldfish genomic DNA.

CHAPTER 8

GENERAL DISCUSSION

8.1 Overview of findings

In order to further understand the mechanisms of macrophage activation in fish, I have described in this thesis the results of experiments designed to; 1) isolate and characterize functional macrophage-like cells from the hemopoietic tissues of different fish species; and 2) biochemically and functionally characterize factors (endogenous and exogenous), which modulated fish macrophage activation. Specifically, my results demonstrated that using previously established protocols for the isolation of primary goldfish macrophages, morphologically and functionally similar macrophage-like cultures were established from carp (*Cyprinus carpio*) kidney leukocytes. In addition, I also established primary monocyte-like cell cultures from the head kidney leukocytes of rainbow trout (*Oncorhynchus mykiss*), which exhibited functional and developmental differences from goldfish and carp primary macrophage-like cultures.

The initial biochemical and functional characterization of mitogen-stimulated goldfish kidney leukocyte supernatants was undertaken by Neumann *et al.,* [318, 322]. These initial studies revealed that 'cytokine-like' activities that exhibited macrophage activating and deactivating properties were present in mitogen-stimulated goldfish kidney supernatants. However, the identification of the native factors responsible for these activities remained unknown. Reported in this thesis are the results of experiments designed to further characterize these factors. Specifically, I have described the partial purification of a native leukocyte-derived goldfish macrophage deactivation factor and based on functional and biochemical analyses, I suggested that this factor may be the teleost homologue of the mammalian deactivating cytokine, TGFβ. In addition, I have demonstrated for the first time that potassium channel activity was required for goldfish macrophage antimicrobial responses. Pertubation of goldfish macrophage potassium channels significantly inhibited the production of ROI and RNI by activated primary goldfish macrophages. This was also related to changes in plasma membrane potential and was similar to reports from studies of mammalian macrophages [569-570]. These findings suggested the importance of ion channels in macrophage activation of lower and higher vertebrates.

Attempts to identify and purify a native goldfish macrophage activating cytokine from crude leukocyte supernatants surprisingly revealed that the serum iron-binding protein transferrin was one of the major modulators of goldfish macrophage activation. Using a combination of liquid chromatography, SDS-PAGE/Western blot analysis, immunopurification, recombinant protein expression, and mass spectrometry analysis, I identified that transferrin cleavage products are potent inducers of the NO response in both goldfish and mammalian macrophages. This serum protein has never been characterized as an inducer of NO production in macrophages from any organism. Therefore, based on current literature, I proposed a model of macrophage activation mediated by this non-cytokine host protein and suggested that transferrin-mediated NO production represents a highly conserved mechanism of macrophage activation. Finally, my results demonstrated that goldfish macrophages expressed TLRs on their surface. These receptors are highly conserved throughout evolution and understanding the roles these receptors play in modulating fish immune responses will significantly enhance our understanding of innate and acquired immune responses in fish.

8.2 Establishment of primary fish macrophage-like cultures

As previously described by Neumann *et al.,* [321] the establishment of primary goldfish *in vitro*-derived kidney macrophage (IVDKM) cultures revealed that goldfish macrophage-like cells secreted autocrine growth factors that mediated their proliferation and differention. A detailed analysis of goldfish IVDKM culture development has subsequently revealed that: 1) goldfish macrophages develop in three distinct phases of growth (i.e. lag-, proliferative-, and senescence-phases); 2) goldfish macrophage-like cells develop from both the differentiation of monocyte-like cells, and direct differentiation from progenitor cells; 3) goldfish macrophages appear to be capable of self-proliferation/renewal; 4) development of goldfish macrophages is associated with specific changes in gene expression; and 5) a unique goldfish soluble colony-stimulating factor-1-like (sCSF-1) receptor has recently been identified in our laboratory (Barreda *et al.*, 2003; submitted for publication) and may play a key role in the regulation of goldfish macrophage proliferation and differentiation. Therefore, the ability to establish primary goldfish macrophage cultures has significantly contributed to a further understanding of monocyte and macrophage developmental processes in fish. In addition, primary goldfish macrophage cultures have allowed us to further delineate the mechanisms of macrophage activation in fish.

In contrast, establishment of primary macrophage cultures from two economically important fish species (carp and rainbow trout) has not been routinely performed. My results (Chapter 3) demonstrated that the same procedures used for establishment of goldfish IVDKM could be successfully adapted for the development of primary monocyte- and macrophage-like cultures from carp kidney leukocytes and primary monocyte-like cultures from the head kidneys of rainbow trout [337, 553]. Carp IVDKM were morphologically and functionally indistinguishable from goldfish IVDKM, which was not surprising due to the close relatedness of these two fish species. The successful establishment of these primary cultures will allow researchers to focus more precisely on monocyte- and macrophage-mediated responses as well as the proliferation and differentiation events of these cells in carp [553]. Previously, studies examining the immunological functions of carp macrophages relied on density gradient enrichment of primary cells. Subsequent adherence to plastic (or glass wool) was then performed for a final enrichment of up to 60% macrophage-like cells [554]. However, the presence of other contaminating leukocytes (i.e. neutrophilic granulocytes) comprised a major impurity, which hindered a definitive conclusion on carp macrophage functional responses. My results show that the development of carp IVDKM could be achieved, which will enable more precise studies (e.g. antimicrobial responses, proliferation, and differentiation events) on these highly enriched macrophage cultures.

I also reported that the incubation of trout head kidney leukocytes in the presence of cell-conditioned medium resulted in the development of proliferating monocyte-like cultures [337]. However, unlike goldfish and carp primary IVDKM cultures, a mature macrophage-like subpopulation was not evident in trout primary kidney monocyte-like cultures (T-PKM). This finding suggested that there were *in vitro* developmental differences between primary cultures established from cyprinids and salmonids. Furthermore, the absence of a mature macrophage-like sub-population in T-PKM may also explain why researchers working with rainbow trout routinely failed to demonstrate the production of NO following treatment with macrophage activating molecules. As discussed in Chapter 3, goldfish and carp primary macrophage culture exhibited the ability to produce NO following stimulation with pathogen and or host-derived factors, which also correlated with the presence of a mature macrophage-like sub-population of cells. In contrast, within T-PKM cultures, only a single functional population of ROI producing monocyte-like cells was observed, and these cells failed to produce NO in response to stimulation. These cultures also did not contain an identifiable mature macrophage-like sub-population. Therefore, using *in vitro* cultivation techniques originally described for establishment of goldfish IVDKM cultures, trout leukocytes failed to develop into mature macrophages and appeared developmentally arrested at the monocyte cell stage. It appears that T-PKM cultures require as yet unknown signals to differentiate fully *in vitro* from ROI producing monocyte-like cells into fully mature (and perhaps NO producing) trout macrophages. Recently, using the procedures described in this thesis, others have attempted to induce the differentiation of trout primary monocytes into macrophages [341]. However, in the absence of cytochemical staining profiles and functional characterization of these cells (i.e. demonstration of NO production), further work is still required to conclusively obtain fully functional *in vitro*-derived trout macrophages.

8.3 Biochemical and functional characterization of goldfish macrophage deactivating factors

The characterization of a mitogen-stimulated goldfish kidney leukocyte derived macrophage deactivation factor (MDF) was reported in this thesis. This factor inhibited the goldfish macrophage NO response in a time-, dose-, and temperature-dependent manner and was capable of inhibiting NO production independent of the activation signal. Following biochemical characterization, an ~ 12 kD protein with a pI < 4.0 was purified that exhibited MDF activity. The molecular identity of this native factor was still unknown, however based on biological activity and biochemical characteriztion, I suggested that this protein was the goldfish homologue of TGFβ (a potent MDF in mammals) [323]. Other researchers have also provided evidence that fish produce TGFβ by the cloning of this cytokine from several fish species [591-593, 600-602]. Furthermore, mammalian TGFβ has been shown to deactivate fish macrophages [327]. While the identification of the goldfish MDF reported in this thesis was not determined, the characterization of a native factor that inhibited goldfish macrophage activation is important. In mammals, the immune response is tightly controlled by balancing the production of cytokines that are responsible for the activation and deactivation of immune cell functions [564]. Imbalances or deficiency in these control mechanisms can often result in susceptibility to diseases or damage of host tissues (i.e. autoimmunity). Therefore, to further understand and potentially modulate immune responses in fish, further work is required to not only identify but also functionally characterize native immunomodulatory factors. Findings reported in this thesis represented a preliminary attempt to characterize native goldfish macrophage modulatory factors.

My results also demonstrated that in addition to native factors, pharmacological blockers of potassium channels also functioned as potent inhibitors of goldfish macrophage antimicrobial responses. This was the first report in teleost that potassium channels were important for immune cell function [579]. Goldfish macrophages treated with potassium channel blockers exhibited a potent inhibition of both ROI and RNI production, which was related to changes in plasma membrane potential (V_m). These findings were also in agreement with our studies in murine macrophages [569-570], and reports by others indicating the importance of potassium channels in macrophage activation [574, 611]. Alterations in the activity of potassium channels caused changes in V_m, which has been shown to have significant effects on proteins and enzymes embedded in the plasma membrane [574, 610]. Modulation of the activities of these proteins would likely significantly alter transduction of signals from the plasma membrane to the nucleus resulting in impaired responses. Elucidation of the mechanisms responsible for macrophage deactivation following blocking of potassium channels would provide further insights into these events. In addition, identification of other factors that alter potassium channel activity in fish (i.e. endogenous factors, environmental factors, or pathogen-derived factors) will be required to fully understand the role of potassium channels in teleost immune cell functions.

8.4 Macrophage activation by transferrin cleavage products

One of the key findings reported in this thesis was the description of a novel function for transferrin. My results demonstrated that following enzymatic cleavage, transferrin activated macrophages, as measured by increased production of reactive nitrogen intermediates. The immunostimulatory transferrin cleavage products were originally purified from mitogen-stimulated goldfish kidney leukocyte supernatants, and the cleaving activity was only observed following leukocyte activation. Furthermore, products released by necrotic fish leukocytes (i.e. macrophages and granulocytes) appeared to play a major role in cleaving transferrin. This interaction between fish leukocytes and a non-cytokine host protein provided a basis for establishing a model for understanding inflammatory processes and the regulation of the immune response of phagocytes in fish. Since macrophage or macrophage-like cells, and iron-binding proteins such as transferrin have been identified in organisms ranging from insects to mammals, this activation pathway may represent a primitive and highly conserved regulator of immune cell functions.

Transferrin is primarily produced by the liver and its major described role is the binding and transport of iron throughout the body [479, 481-483]. Consequently, the iron-binding property of this serum protein creates a bacteriostatic environment by limiting the availability of this essential element for pathogens. A review of the literature also suggests that this protein exhibits a variety of other biological activities (independent of iron-binding), which includes growth and proliferation factor-like activities [493-495], induction of chemokine synthesis [497], ability to bind other proteins [499], and direct bacterialcidal effects [503]. Recent evidence in chickens has also demonstrated that ovatransferrin was a key activator of immune cells [501] [502], and in fish, transferrin is an acute phase protein [160] that activates macrophages (this thesis). Therefore, transferrin is a multifunctional molecule that plays a key role in binding iron as well as the modulation of immune cell activities.

8.4.1 Mechanisms for increasing transferrin concentrations at inflammatory sites

In order to exert immunomodulatory functions, it is essential that a protein is rapidly available at the infection/inflammatory site. There are two processes that could potentially facilitate an increased concentration of transferrin at these sites: 1) transferrin secreted by activated immune cells such as macrophages; and 2) during inflammatory processes, increased vascular permeability caused the leakage of serum transferrin into the inflammatory site. I have demonstrated that following stimulation with pathogen and/or host-derived factors, activated goldfish macrophages exhibited an up-regulation of transferrin mRNA expression. This suggested that goldfish macrophages may produce transferrin following activation. In mammals, IFNγ-stimulated macrophages produced transferrin, as well do T cells [181, 655-656]. Therefore, at infection/inflammatory sites, activated immune cells contribute to the increased availability of this immunomodulatory protein. Secondly, progression of inflammatory responses can lead to increased vascular permeability. The events leading to vessel leakage are complex and controlled by a range of inflammatory mediators, which themselves are regulated by a complex process involving gene expression, transcription and translation events, secretion of proteins, and endothelial cell activation. A detailed description of all of the events culminating in increased vascular permeability is beyond the scope of this discussion. However, it is well established that vascular permeability causes a significant increase in the levels of transferrin during early stages of inflammation [505-508]. In fact, transferrin is often used as the primary marker for the transudation of serum proteins during inflammatory processes [505, 507-508, 704]. In goldfish, intraperitoneal injection of sterile thioglycollate induces a typical pattern of inflammation characterized by intraperitoneal exudation of serum proteins followed by influx of macrophages and heterophils [509]. Although the exudation of transferrin was not specifically monitored in that study. Evans Blue staining indicated that the levels of serum proteins peaked 30 min after the induction of inflammation. Therefore, during inflammation, increased concentrations of transferrin could be rapidly increased by a combination of localized production by activated immune cells and increased transudation through leaky vessels. However, the time-course and relative contributions of these processes for mobilizing transferrin to inflammatory sites is unknown.

8.4.2 Mechanism for the generation of transferrin cleavage products

The immunostimulatory activity of transferrin was only observed following cleavage of the native molecule into smaller fragments. As previously mentioned, this process was mediated by stimulated goldfish kidney leukocytes and products released from damaged/lysed goldfish leukocytes. Therefore, while increased production by activated immune cells and/or transudation through leaky vessels increased the concentration of transferrin at inflammatory sites, the cleavage of transferrin was mediated by factors derived from activated leukocytes. For example, secretion of human leukocyte elasase was in part responsible for the pathogenesis associated with bronchiectasis, bronchitis, acute respiratory distress syndrome, emphysema, and cystic fibrosis [669-670]. Of particular interest was the case of cystic fibrosis where both pathogen- and host cell-derived proteases resulted in the generation of transferrin cleavage products. A number of neutrophil- and bacteria-derived proteases have been reported to alter the physiochemical nature of transferrin and lactoferrin at sites of *Pseudomonas aeruginosa* infection [673-674]. Protease cleavage of iron-binding proteins was believed to contribute to tissue injury by increasing the availability of iron to replicating pathogens and also increasing the potential for local hydroxyl radical formation [675-677]. Under normal physiological conditions, proteinase inhibitors would likely counterbalance the effects of proteolytic enzymes [705]. However, during inflammation, increased concentrations of enzymes (i.e. from degranulating neutrophils or those produced by pathogens) may degrade these regulatory protease inhibitors [705-708]. This would explain why transferrin fragments are present in the bronchoalveolar fluids of cytic fibrosis patients [674]. Furthermore, pathogen-derived elastase has been shown to degrade other plasma proteins as well as extracellular products from fibroblasts (e.g. complement C3, antiproteinases, kininogens, and proteoglycans), which are believed to contribute to the pathogenisis of chronic ulcers [709]. These findings suggested that endogenous proteins such as transferrin were susceptible to cleavage at inflammatory sites, which supports my observation that transferrin cleavage products were present in mitogen-stimulated goldfish kidney leukocyte supernatants. In addition, I also reported that elastase cleaved transferrin into smaller fragments, which correlated with the induction of NO in goldfish macrophages. Combined with its ability to degrade endogenous proteins, pancreatic elastase has recently been shown to be a mannose-binding protein capable of inducing systemic inflammation [710].

Degradation of host proteins like transferrin may not be entirely detrimental to the host. Although both transferrin and lactoferrin were degraded during inflammation, this may under certain circumstances be benefitial to the host. Treatment of lactoferrin with pepsin generated a smaller peptide known as lactoferricin. Lactoferricin was initially identified as an antimicrobial peptide that functioned as a multifunctional innate-defense protein in milk (reviewed by [711]). Subsequently, lactoferricin has been shown to exhibit immunostimulatory activities in several infection models in mice and was also shown to elicit a direct antitumor effect [711-712]. Identified within the N-lobe of transferrin was a sequence encoding an insect defensin, and isolation of this transferrin-derived peptide demonstrated that it exhibited bacterialcidal properties [503]. Therefore, following cleavage, transferrin may also function as an antibacterial peptide. Finally, generation of transferrin cleavage products may provide a pro-inflammatory signal for the activation of macrophages and generation of antimicrobial molecules such as NO (this thesis).

8.4.3 Transferrin-mediated macrophage activation: proposed mechanism and potential receptor

How macrophages perceive transferrin cleavage products as a pro-inflammatory signal remains to be elucidated. However, there is growing evidence in mammals that the immune system is not necessarily concerned with recognition of non-self pathogen-derived products (i.e. LPS) as it is with recognizing tissue damage, which may contain both pathogen-derived and endogenous signals. Many of these endogenous signals are not produced by healthy cells but are generated or modified by distressed or injured tissues during inflammatory responses. Examples of endogenous proteins capable of initiating immune responses include fibrinogen [10], fibronectin [11], surfactant protein-A [152], heat shock proteins [153-154], hyaluronan [155], and heparan sulphate [156]. All of these endogenous host molecules exhibit macrophage activation activities, and are thought to be the endogenous ligands of TLRs. These endogenous ligands have also been defined as 'danger/alarm' signals (e.g. molecules produced by stressed cells, or products that are usually found inside healthy cells), which is the basis of the 'danger' model of immune activation. This model proposed that the immune system does not detect foreign material *per se* but rather detects cellular injury caused by infections and other environmental stresses [621-622]. Recently, this original model has been expanded into what it now called the 'surveillance' model of immune activation [151]. This model proposed that immune cells (i.e. macrophages) recognized products released from necrotic cells, pathogen-derived factors (i.e. LPS), and fragments derived from the degradation of endogenous macromolecules. Therefore, my hypothesis is that transferrin cleavage products function as an endogenous 'danger' signal capable of initiating the production of NO by macrophages. I also demonstrated that transferrin fragments can activate mammalian macrophages and that a potential cleavage-site is shared between transferrin from a variety of different species. These findings suggested that transferrin-mediated macrophage activation is a conserved mechanism in vertebrates and perhaps in invertebrates where iron transport proteins homologous to transferrin have also been identified [488-490, 630].

Studies in insects have also provided clues into immune processes in mammals and may help to further elucidate the mechanism of transferrin-mediated macrophage activation. A candidate receptor that I believe is responsible for recognizing cleaved transferrin fragments belongs to the highly conserved family of Toll-like receptors that were originally identified as key mediators of development in *Drosophila* [116]. These receptors were found to also initiate innate antifungal responses in the fly, and since this discovery, a family of TLRs have been identified in fish, birds, and mammals [158-160, 695-696]. The mechanism of Toll receptor activation in *Drosophila* also required the presence of enzymes and an endogenous protein called Spaetzle. A proteolytically cleaved form of *Drosophila* Spatzle was identified as the extracellular ligand of Toll in both embryonic development and initiation of immune response pathways [9, 713]. Subsequently, activation of immune responses in the fly was shown to be dependent on the initiation of a blood serine protease [667], which was also inhibited by a serpin [668]. In mammals, LPS-mediated activation of monocytes was blocked by the serpin, antithrombin III [157], which suggests that in response to pathogens, activation of extracellular serine proteases in invertebrates and vertebrates was required for initiation of immune responses. Therefore, conserved innate immune receptors like Toll (and TLRs), initiation of protease cascades, and cleavage of endogenous proteins may all be conserved components of immune cell activation.

In goldfish, I have identified a macrophage TLR, demonstrated that specific enzymes are necessary for initiation of macrophage activation, and have purified transferrin cleavage products that induced macrophage activation. However, a direct interaction between these immunostimulatory transferrin cleavage products and binding/activation of a goldfish macrophage TLR has not yet been established in fish or mammals. It appears however that goldfish macrophages are capable of recognizing and responding to a variety of different pathogens (Fig. 8.1), which may be mediated by the presence of different TLRs. Interestingly, the induction of NO production in goldfish macrophages by different pathogens was significantly enhanced by the addition of transferrin, which further indicated the importance of this endogenous protein in the activation of goldfish macrophages. In Figure 8.2, I have summarized the potential sources of transferrin and different mechanisms resulting in the cleavage of this protein during infection and/or inflammation, which have been discussed in throughout this chapter. Interestingly, it has been recently reported that β-Defensin 2 can activate dendritic cells, which was dependent on TLR-4 [716]. Defensins are small antimicrobial peptides that are characterized by the presence of 6 conserved cysteine residues and are produced in response to microbial infection. As described earlier (section 1.7), a defensin-like domain was identified within the N-lobe domain of chicken ovatransferrin [503]. This raises the intriguing possibility that degradation of transferrin can expose and/or release a defensin-like peptide to directly kill microbes, but which may also activate immune cells by binding to TLRs. Experiments designed to confirm this proposed mechanism would help to further elucidate how transferrin cleavage products activate macrophages.

Precisely how transferrin cleavage products induce macrophage activation is still unknown, but initial evidence suggests that iron-binding proteins are recognized by goldfish macrophage TLRs. In preliminary experiments, I have cloned the region of the goldfish macrophage TLR that encoded for the LRR extracellular ligand-binding domain into the same expression vector used to produce recombinant goldfish transferrin. Following expression of the recombinant LRR domain (~90 kD), it was consistently observed that three other proteins co-purified with the recombinant receptor (~40 kD, ~30 kD, and ~25 kD; Fig. 8.3). Only the ~90 kD protein was recognized by a monoclonal anti-V5 antibody confirming its identification as the recombinant goldfish LRR domain. The other proteins were analyzed by mass spectrometry and subjected to tryptic digestion mapping for identification. The ~40 kD protein remains unidentified, however, the ~30 kD and ~25 kD proteins were identified as ferritin sub-units. Interestingly, like transferrin, ferritin is an iron-binding protein responsible for the storage of iron, however it is an intracellular protein. The ferritin sub-units that co-purified with goldfish rLRR domain were also identified as insect ferritin, which undoubtedly were derived from the insect cells (Sf9) used for eukaryotic protein expression. Since the cells were cultured in serum-free insect medium, no transferrin was available for potential binding. However these preliminary findings suggested that the goldfish macrophage TLR binds an iron-binding protein. The fact that insect ferritin was recognized by this goldfish receptor further reinforces the potential conservation of this proposed mechanism. Future studies are required to establish a direct interaction between TLRs and iron-binding proteins such as transferrin and ferritin.

8.5 Future research

I have used a combination of biological and molecular approaches to further elucidate the mechanisms of macrophage activation in fish. The use of molecular techniques such as cDNA library screening, degenerative PCR, and sequencing of the Fugu genome, has enabled the identification of a variety of different teleost cytokines and interleukins, which share significant homology with mammalian factors (i.e. IL-1β, IL-8, TNF-α, TGF-β, and IFN). However, while the genes encoding these factors have been cloned and sequenced, there are still relatively few studies that focus on the characterization of native teleost immunomodulatory factors.

In order to functionally characterize native teleost immunomodulatory factors, it is important to first obtain fuctional immune cells for the development of immunological bioassays. Using procedures originally designed for the isolation and cultivation of primary goldfish macrophage cultures, I have shown that primary macrophage cultures can also be derived from other fish species (i.e. carp and rainbow trout). The proliferation of these cultures appeared to be dependent on the production of autocrine growth factors and the purification of these fish macrophage growth factors is essential for developing future culture techniques that may allow for the selection of specific macrophage sub-populations from these heterogeneous cultures. Further characterization (i.e. developmental and functional) of these sub-populations will also advance our understanding of fish macrophage biology. In addition, there is virtually no specific surface marker antigens identified or monoclonal antibodies available to aid in further characterizing heterogeneous macrophage cultures. Therefore future research should also focus on identifying specific genes expressed by various teleost immune cells, which would then facilitate the identification of pathways leading to macrophage development in teleost and the subsequent isolation of specific macrophage sub-populations.

In an attemp to purifiy a native fish macrophage activation factor, I identified that transferrin cleavage products induced the activation of goldfish and mammalian macrophages. Furthermore, it appears that this represented a conserved mechanism of macrophage activation that is likely mediated by innate immune receptors such as TLRs. It is therefore essential that future research confirm that goldfish and mammalian macrophage TLRs are capable of: 1) binding transferrin cleavage products; and 2) that this receptor-ligand interaction causes macrophage activation. Production of the recombinant ligand (i.e. transferrin cleavage products) and the recombinant receptor (i.e. TLR), followed by protein-protein interactions studies will confirm whether transferrin cleavage products bind TLRs or whether other receptors are involved. Furthermore, in fish limited information is available regarding signal transduction pathways following ligation of macrophage immune receptors. A detailed analysis of the intracellular signaling events responsible for fish macrophage activation by ligation of TLR should be explored

In mammals, TLRs have been extensively studied and a wide variety of endogenous ligands of these receptors have been identified. These receptors have also significantly advanced our understanding of innate and adaptive immune processes in mammals. In teleost no information is available regarding the specific ligands for various TLRs and the identification of a goldfish macrophage TLR will allow for the identification of other teleost TLRs and their ligands. Studies designed to characterize the roles of teleost TLRs will significantly contribute to further understanding innate (and perhaps adaptive) immune responses in fish. For example, mutations in a specific TLR may be responsible for increased susceptibilities of fish to the pathogens recognized by this receptor. Identification of the mutations and subsequent screening of fish populations may enable not only the prediction but also the prevention of disease outbreaks in aquaculture settings.

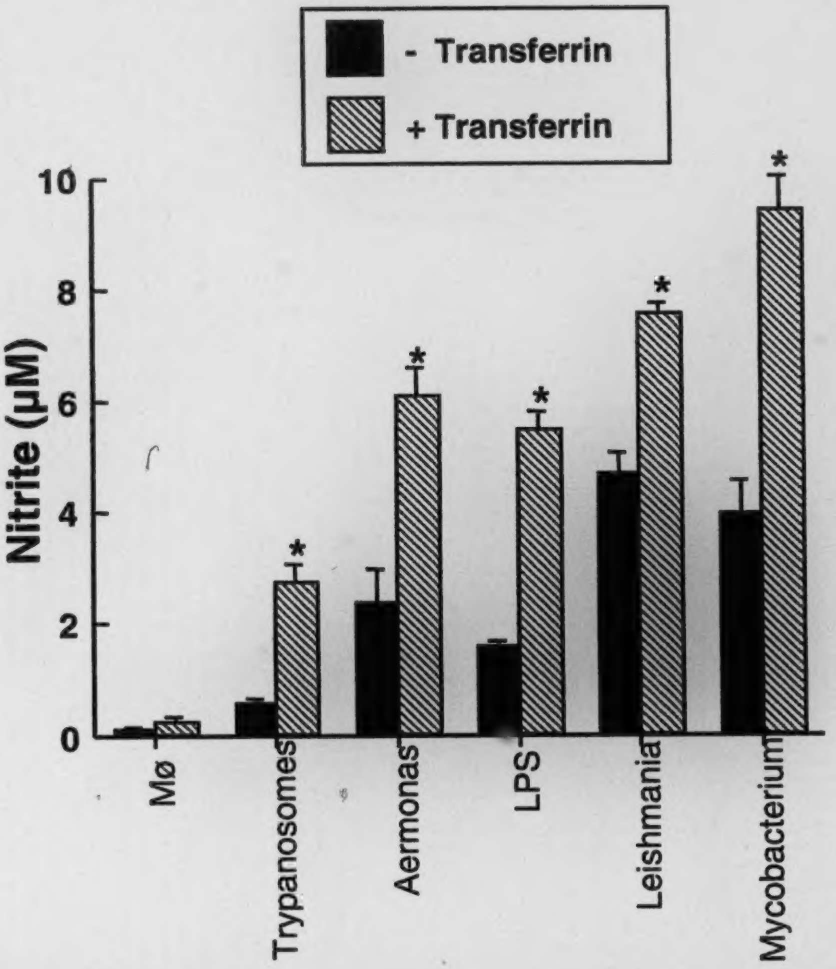

Figure 8.1. Addition of transferrin to goldfish macrophages exposed to different pathogens or pathogen products significantly increased production of nitric oxide. Goldfish macrophages (5×10^4 cells/well) were seeded in triplicate into 96-well half-area tissue culture plates and stimulated with heat-killed *T. danilewski* (10:1 parasite to macrophage ratio), heat-killed *A. salmonicida* (1:200 dilution of heat-killed stock), LPS (1 µg/ml), *L. major* promastigotes (10:1), or *M. chelonei* (10:1). Stimulated cells were simultaneously incubated in medium alone (black bars), or in medium supplemented with 7.5 µg/well transferrin (thatched bars). Macrophages were then incubated for 72 h at 20°C prior to determination of NO production. Each bar represents the mean ± SEM of triplicate cultures and results are from a representative experiment of three individual experiments that were performed. * $P<0.0001$ compared with nitrate produced by cells incubated in the absence of 7.5 µg/well of transferrin.

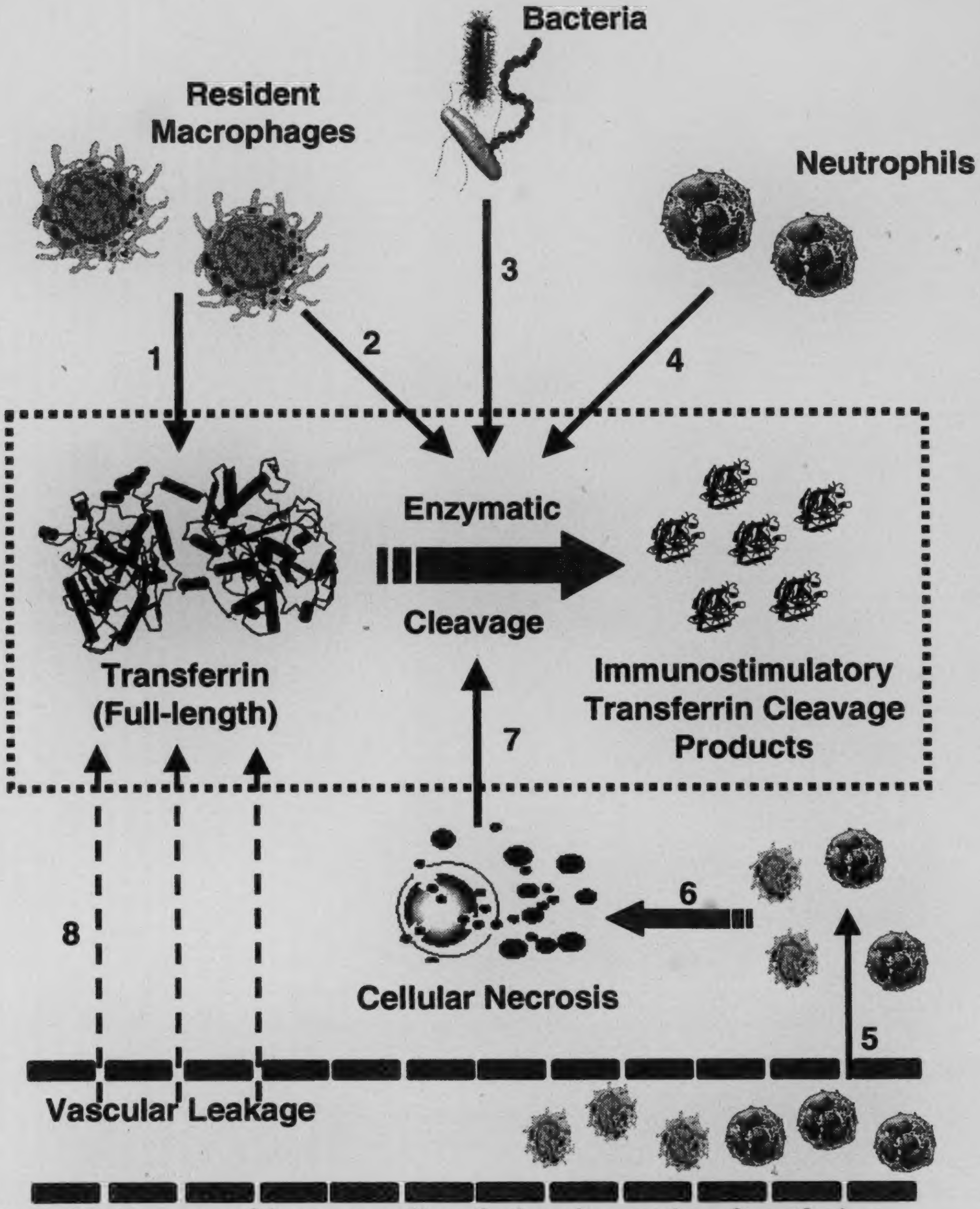

Figure 8.2. Summary of the sources of transferrin and generation of transferrin cleavage products during infections/inflammation. Transferrin is produced by activated macrophages (1) and/or derived from serum due to vascular leakage (8). Cleavage of the native transferrin is mediated by enzymes released from activated macrophages (2), pathogen-derived enzymes (3), and degranulating neutrophils (4). In addtion influx of leukocytes into the inflammatory lesion (5) and subsequent necrosis (7) causes the release of factor with transferrin-cleaving activity.

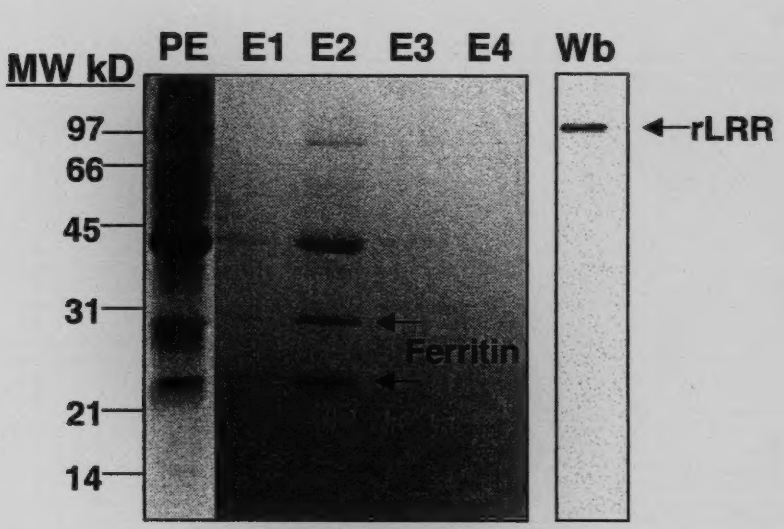

Figure 8.3. Co-purification of insect ferritin with the recombinant leucine-rich repeat domain (rLRR) of a goldfish macrophage TLR. Concentrated crude supernatants from Sf9 cells transfected with a rLRR expression construct were separated by SDS-PAGE (reducing) prior to application to a nickel column (PE). Bound proteins were eluted from the nickle column with 100 mM imidazole in 1 ml fractions (E1 to E4). A Western blot using the anti-V5 monoclonal antibody was also performed for detection of the recombinant protein (Wb). Three major protein bounds (~40 kD, ~30 kD, and ~25 kD) co-purified with the recombinant protein (~90 kD), and the two smaller bands were identified as insect ferritin subunits by mass spectrometry (indicated by arrows).

CHAPTER 9

REFERENCES

1. Gumulak-Smith J., Teachman A., Tu A.H., Simecka J.W., Lindsey J.R., Dybvig K. (2001) Variations in the surface proteins and restriction enzyme systems of *Mycoplasma pulmonis* in the respiratory tract of infected rats. Molecuar Microbiology. **40**: 1037.
2. Nickel R., Ott C., Dandekar T., Leippe M. (1999) Pore-forming peptides of *Entamoeba dispar*. Similarity and divergence to amoebapores in structure, expression and activity. European Journal of Biochemistry. **265**: 1002.
3. Vaughan R.B. (1965) The romantic rationalist: a study of Elie Metchnikoff. Medical History. **9**: 201.
4. Medzhitov R., Janeway C.A.J. (1997) Innate immunity: the virtues of a nonclonal system of recognition. Cell. **91**: 295.
5. Medzhitov R., Janeway C.J. (2000) Innate immune recognition: mechanisms and pathways. Immunology. **173**: 89.
6. Medzhitov R. (2001) Toll-like receptors and innate immunity. Nature Reviews Immunology. **1**: 135.
7. Medzhitov R., Janeway C.A.J. (1997) Innate immunity: impact on the adaptive immune response. Current Opinion inImmunology. **9**: 4.
8. Landmann R., Muller B., Zimmerli W. (2000) CD14, new aspects of ligand and signal diversity. Microbes and Infection. **2**: 295.
9. Lemaitre B., Nicolas E., Michaut L., Reichhart J.M., Hoffmann J.A. (1996) The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell. **86**: 973.
10. Smiley S.T., King J.A., Hancock W.W. (2001) Fibrinogen stimulates macrophage chemokine secretion through Toll-like receptor 4. Journal of Immunology. **167**: 2887.
11. Okamura Y., Watari M., Jerud E.S., Young D.W., Ishizaka S.T., Rose J., Chow J.C., Strauss I., J. F. (2001) The extra domain A of fibronectin activates Toll-like receptor 4. Journal of Biological Chemistry. **276**: 10229.
12. Linehan S.A., Martinez-Pomares L., Gordon S. (2000) Macrophage lectins in host defence. Microbes and Infection. **2**: 279.
13. Drickamer K., Taylor M.E. (1993) Biology of animal lectins. Annual Review of Cellular Bioliogy. **9**: 237.
14. Weis W.I., Taylor M.E., Drickamer K. (1998) The C-type lectin superfamily in the immune system. Immunological Reviews. **163**: 19.
15. Fraser I.P., Koziel H., Ezekowitz R.A. (1998) The serum mannose-binding protein and the macrophage mannose receptor are pattern recognition molecules that link innate and adaptive immunity. Seminars in Immunology. **10**: 363.
16. Ezekowitz R.A., Sastry K., Bailly P., Warner A. (1990) Molecular characterization of the human macrophage mannose receptor: demonstration

of multiple carbohydrate recognition-like domains and phagocytosis of yeasts in Cos-1 cells. Journal of Experimental Medicine. **172**: 1785.

17. Ezekowitz R.A., Williams D.J., Koziel H., Armstrong M.Y., Warner A., Richards F.F., Rose R.M. (1991) Uptake of *Pneumocystis carinii* mediated by the macrophage mannose receptor. Nature. **351**: 155.
18. Feizi T. (2000) Carbohydrate-mediated recognition systems in innate immunity. Immunological Reviews. **173**: 79.
19. Ikeda K., Sannoh T., Kawasaki N., Kawasaki T., Yamashina I. (1987) Serum lectin with known structure activates complement through the classical pathway. Journal of Biological Chemistry. **262**: 7451.
20. Schweinle J.E., Ezekowitz R.A., Tenner A.J., Kuhlman M., A. J.K. (1989) Human mannose-binding protein activates the alternative complement pathway and enhances serum bactericidal activity on a mannose-rich isolate of *Salmonella*. Journal of Clinical Investigation. **84**: 1821.
21. Turner M.W. (1996) Mannose-binding lectin: the pluripotent molecule of the innate immune system Immunology Today. **17**: 532.
22. Gadjeva M., Thiel S., Jensenius J.C. (2001) The mannan-binding-lectin pathway of the innate immune response. Current Opinion in Immunology. **13**: 74.
23. Turner M.W. (1998) Mannose-binding lectin (MBL) in health and disease, Immunobiology. **199**: 327.
24. Devyatyarova-Johnson M., Rees I.H., Robertson B.D., Turner M.W., Klein N.J., Jack D.L. (2000) The lipopolysaccharide structures of *Salmonella enterica* serovar *Typhimurium* and *Neisseria gonorrhoeae* determine the attachment of human mannose-binding lectin to intact organisms. Infection and Immunity. **68**: 3894.
25. Matsushita M., Fujita T. (1992) Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease. Journal of Experimental Medicine. **176**: 1497.
26. Thiel S., Vorup-Jensen T., Stover C.M., Schwaeble W., Laursen S.B., Poulsen K., Willis A.C., Eggleton P., Hansen S., Holmskov U., Reid K.B., Jensenius J.C. (1997) A second serine protease associated with mannan-binding lectin that activates complement. Nature. **386**: 506.
27. Garred P., Voss A., Madsen H.O., Junker P. (2001) Association of mannose-binding lectin gene variation with disease severity and infections in a population-based cohort of systemic lupus erythematosus patients. Genes and Immunity. **2**: 242.
28. Peterslund N.A., Koch C., Jensenius J.C., Thiel S. (2001) Association between deficiency of mannose-binding lectin and severe infections after chemotherapy. Lancet. **358**: 637.
29. Neth O., Hann I., Turner M.W., Klein N.J. (2001) Deficiency of mannose-binding lectin and burden of infection in children with malignancy: a prospective study. Lancet. **358**: 614.
30. Alan R., Ezekowitz B. (2001) Mannose-binding lectin in prediction of susceptibility to infection. Lancet. **358**: 598.

31. Stahl P.D., Ezekowitz R.A. (1998) The mannose receptor is a pattern recognition receptor involved in host defense. Current Opinion in Immunology. **10**: 50.
32. Takahashi K., Donovan M.J., Rogers R.A., Ezekowitz R.A. (1998) Distribution of murine mannose receptor expression from early embryogenesis through to adulthood. Cell and Tissue Research. **292**: 311.
33. Taylor M.E., Conary J.T., Lennartz M.R., Stahl P.D., Drickamer K., J. (1991) Primary structure of the mannose receptor contains multiple motifs resembling carbohydrate-recognition domains. Journal of Biological Chemistry. **265**: 12156.
34. Harris N., Super M., Rits M., Chang G., Ezekowitz R.A. (1992) Characterization of the murine macrophage mannose receptor: demonstration that the downregulation of receptor expression mediated by interferon-gamma occurs at the level of transcription. Blood. **80**: 2363.
35. Stahl P.D., Rodman J.S., Miller M.J., Schlesinger P.H. (1978) Evidence for receptor-mediated binding of glycoproteins, glycoconjugates, and lysosomal glycosidases by alveolar macrophages. Proceedings of the National Academy of Sciences of the United States of America.**75**: 1399.
36. Shepherd V.L., Lee Y.C., Schlesinger P.H., Stahl P.D. (1981) L-Fucose-terminated glycoconjugates are recognized by pinocytosis receptors on macrophages. Proceedings of the National Academy of Sciences of the United States of America.**78**: 1019.
37. Marodi L., Korchak H.M., Johnston R.B., Jr. (1991) Mechanisms of host defense against *Candida* species. I. Phagocytosis by monocytes and monocyte-derived macrophages. Journal of Immunology. **146**: 2783.
38. Schlesinger L.S. (1993) Macrophage phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. Journal of Immunology. **150**: 2920.
39. Schlesinger L.S., Hull S.R., Kaufman T.M. (1994) Binding of the terminal mannosyl units of lipoarabinomannan from a virulent strain of *Mycobacterium tuberculosis* to human macrophages. Journal of Immunology. **152**: 4070.
40. Athamna A., Ofek I., Keisari Y., Markowitz S., Dutton G.G., Sharon N. (1991) Lectinophagocytosis of encapsulated *Klebsiella pneumoniae* mediated by surface lectins of guinea pig alveolar macrophages and human monocyte-derived macrophages. Infection and Immunity. **59**: 1673.
41. Kabha K., Nissimov L., Athamna A., Keisari Y., Parolis H., Parolis L.A., Grue R.M., Schlepper-Schafer J., Ezekowitz A.R., Ohman D.E. (1995) Relationships among capsular structure, phagocytosis, and mouse virulence in *Klebsiella pneumoniae*. Infection and Immunity. **63**: 847.
42. Speert D.P., Wright S.D., Silverstein S.C., Mah B. (1988) Functional characterization of macrophage receptors for *in vitro* phagocytosis of unopsonized *Pseudomonas aeruginosa*. Journal of Clinical Investigation. **82**: 872.
43. Blackwell J.M., Ezekowitz R.A., Roberts M.B., Channon J.Y., Sim R.B., Gordon S., J. (1985) Macrophage complement and lectin-like receptors bind

Leishmania in the absence of serum. Journal of Experimental Medicine. **162**: 324.

44. Peters C., Aebischer T., Stierhof Y.D., Fuchs M., Overath P., J. (1995) The role of macrophage receptors in adhesion and uptake of *Leishmania mexicana* amastigotes. Journal of Cell Science. **108 (Pt 12)**: 3715.
45. Kahn S., Wleklinski M., Aruffo A., Farr A., Coder D., Kahn M., J. (1995) *Trypanosoma cruzi* amastigote adhesion to macrophages is facilitated by the mannose receptor. Journal of Experimental Medicine. **182**: 1243.
46. Marodi L., Forehand J.R., Johnston R.B., Jr. (1991) Mechanisms of host defense against *Candida* species. II. Biochemical basis for the killing of *Candida* by mononuclear phagocytes. Journal of Immunology. **146**: 2790.
47. Yamamoto Y., Klein T.W., Friedman H. (1997) Involvement of mannose receptor in cytokine interleukin-1beta (IL- 1beta), IL-6, and granulocyte-macrophage colony-stimulating factor responses, but not in chemokine macrophage inflammatory protein 1beta (MIP-1beta), MIP-2, and KC responses, caused by attachment of *Candida albicans* to macrophages. Infection and Immunity. **65**: 1077.
48. Ohsumi Y., Lee Y.C. (1987) Mannose-receptor ligands stimulate secretion of lysosomal enzymes from rabbit alveolar macrophages. Journal of Biological Chemistry. **262**: 7955.
49. Berton G., Gordon S. (1983) Modulation of macrophage mannosyl-specific receptors by cultivation on immobilized zymosan. Effects on superoxide-anion release and phagocytosis. Immunology. **79**: 705.
50. Rouzer C.A., Scott W.A., Hamill A.L., Cohn Z.A. (1982) Synthesis of leukotriene C and other arachidonic acid metabolites by mouse pulmonary macrophages. Journal of Experimental Medicine. **155**: 720.
51. Stein M., Gordon S. (1991) Regulation of tumor necrosis factor (TNF) release by murine peritoneal macrophages: role of cell stimulation and specific phagocytic plasma membrane receptors. European Journal of Immunology. **21**: 431.
52. Garner R.E., Rubanowice K., Sawyer R.T., Hudson J.A. (1994) Secretion of TNF-alpha by alveolar macrophages in response to *Candida albicans* mannan. Journal of Leukocyte Biology. **55**: 161.
53. Shibata Y., Metzger W.J., Myrvik Q.N. (1997) Chitin particle-induced cell-mediated immunity is inhibited by soluble mannan: mannose receptor-mediated phagocytosis initiates IL-12 production. Journal of Immunology. **158**: 2462.
54. Murai M., Aramaki Y., Tsuchiya S. (1995) Contribution of mannose receptor to signal transduction in Fc gamma receptor-mediated phagocytosis of mouse peritoneal macrophages induced by liposomes. Journal of Leukocyte Biology. **57**: 687.
55. Murai M., Aramaki Y., Tsuchiya S. (1996) Alpha 2-macroglobulin stimulation of protein tyrosine phosphorylation in macrophages via the mannose receptor for Fc gamma receptor-mediated phagocytosis activation. Immunology. **89**: 436.

56. Marodi L., Schreiber S., Anderson D.C., MacDermott R.P., Korchak H.M., Johnston R.B., Jr. (1993) Enhancement of macrophage candidacidal activity by interferon-gamma. Increased phagocytosis, killing, and calcium signal mediated by a decreased number of mannose receptors. Journal of Clinical Investigation. **91**: 2596.
57. Ross G.D. (1989) Complement and complement receptors. Current Opinion in Immunology. **2**: 50.
58. Stewart M., Thiel M., Hogg N. (1995) Leukocyte integrins. Current Opinion in Cell Biology. **7**: 690.
59. Law S.K., Gagnon J., Hildreth J.E., Wells C.E., Willis A.C., Wong A.J. (1987) The primary structure of the beta-subunit of the cell surface adhesion glycoproteins LFA-1, CR3 and p150,95 and its relationship to the fibronectin receptor. EMBO Journal. **6**: 915.
60. Kishimoto T.K., O'Connor K., Lee A., Roberts T.M., Springer T.A. (1987) Cloning of the beta subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family. Cell. **48**: 681.
61. Springer T.A., Dustin M.L., Kishimoto T.K., Marlin S.D. (1987) The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. Annual Review of Immunology. **5**: 223.
62. Wright S.D., Rao P.E., Van Voorhis W.C., Craigmyle L.S., Iida K., Talle M.A., Westberg E.F., Goldstein G., Silverstein S.C. (1983) Identification of the C3bi receptor of human monocytes and macrophages by using monoclonal antibodies. Proceedings of the National Academy of Sciences of the United States of America. **80**: 5699.
63. Wright S.D., Levin S.M., Jong M.T., Chad Z., Kabbash L.G. (1989) CR3 (CD11b/CD18) expresses one binding site for Arg-Gly-Asp-containing peptides and a second site for bacterial lipopolysaccharide. Journal of Experimental Medicine. **169**: 175.
64. Ross G.D., Medof M.E. (1985) Membrane complement receptors specific for bound fragments of C3. Advances in Immunology. **37**: 217.
65. Talamas-Rohana P., Wright S.D., Lennartz M.R., Russell D.G. (1990) Lipophosphoglycan from *Leishmania mexicana* promastigotes binds to members of the CR3, p150,95 and LFA-1 family of leukocyte integrins. Journal of Immunology. **144**: 4817.
66. Bullock W.E., Wright S.D. (1987) Role of the adherence-promoting receptors, CR3, LFA-1, and p150,95, in binding of *Histoplasma capsulatum* by human macrophages. Journal of Experimental Medicine. **165**: 195.
67. Payne N.R., Horwitz M.A. (1987) Phagocytosis of *Legionella pneumophila* is mediated by human monocyte complement receptors. Journal of Experimental Medicine. **166**: 1377.
68. Schlesinger L.S., Horwitz M.A. (1991) Phagocytosis of *Mycobacterium leprae* by human monocyte-derived macrophages is mediated by complement receptors CR1 (CD35), CR3 (CD11b/CD18), and CR4 (CD11c/CD18) and

IFN-gamma activation inhibits complement receptor function and phagocytosis of this bacterium. Journal of Immunology. **147**: 1983.

69. Brittingham A., Morrison C.J., McMaster W.R., McGwire B.S., Chang K.P., Mosser D.M. (1995) Role of the *Leishmania* surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. Journal of Immunology. **155**: 3102.
70. Haworth R., Platt N., Keshav S., Hughes D., Darley E., Suzuki H., Kurihara Y., Kodama T., Gordon S. (1997) The macrophage scavenger receptor type A is expressed by activated macrophages and protects the host against lethal endotoxic shock. Journal of Experimental Medicine. **186**: 1431.
71. Pearson A.M. (1996) Scavenger receptors in innate immunity. Current Opinion in Cell Biology. **8**: 20.
72. Brown M.S., Goldstein J.L. (1983) Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. Annual Review of Biochemistry. **52**: 223.
73. Krieger M., Herz J. (1994) Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). Annual Review of Biochemistry. **63**: 601.
74. Abrams J.M., Lux A., Steller H., Krieger M. (1992) Macrophages in *Drosophila* embryos and L2 cells exhibit scavenger receptor-mediated endocytosis. Proceedings of the National Academy of Sciences of the United States of America. **89**: 10375.
75. Pearson A., Lux A., Krieger M. (1995) Expression cloning of dSR-CI, a class C macrophage-specific scavenger receptor from *Drosophila melanogaster*. Proceedings of the National Academy of Sciences of the United States of America. **92**: 4056.
76. Dunne D.W., Resnick D., Greenberg J., Krieger M., Joiner K.A. (1994) The type I macrophage scavenger receptor binds to gram-positive bacteria and recognizes lipoteichoic acid. Proceedings of the National Academy of Sciences of the United States of America. **91**: 1863.
77. Hampton R.Y., Golenbock D.T., Penman M., Krieger M., Raetz C.R. (1991) Recognition and plasma clearance of endotoxin by scavenger receptors. Nature. **352**: 342.
78. Fraser I., Hughes D., Gordon S. (1993) Divalent cation-independent macrophage adhesion inhibited by monoclonal antibody to murine scavenger receptor. Nature. **364**: 343.
79. Hughes D.A., Fraser I.P., Gordon S. (1995) Murine macrophage scavenger receptor: in vivo expression and function as receptor for macrophage adhesion in lymphoid and non-lymphoid organs. European Journal of Immunology. **25**: 466.
80. Platt N., Suzuki H., Kurihara Y., Kodama T., Gordon S. (1996) Role for the class A macrophage scavenger receptor in the phagocytosis of apoptotic thymocytes *in vitro*. Proceedings of the National Academy of Sciences of the United States of America. **93**: 12456.

81. Ibrahim M.A., Chain B.M., Katz D.R. (1995) The injured cell: the role of the dendritic cell system as a sentinel receptor pathway. Immunology Today. **16**: 181.

82. Bell M.D., Lopez-Gonzalez R., Lawson L., Hughes D., Fraser I., Gordon S., Perry V.H. (1994) Upregulation of the macrophage scavenger receptor in response to different forms of injury in the CNS. Journal of Neurocytology. **23**: 605.

83. Davie E.W., Fujikawa K., Kisiel W. (1991) The coagulation cascade: initiation, maintenance, and regulation. Biochemistry. **30**: 10363.

84. Stern D. M., Esposito C., Gerlach H., Gerlach M., Ryan J., Handley D., Nawroth P. (1991) Endothelium and regulation of coagulation. Diabetes Care. **14**: 1660

85. Kodama T., Freeman M., Rohrer L., Zabrecky J., Matsudaira P., Krieger M. (1990) Type I macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils. Nature. **343**: 531.

86. Rohrer L., Freeman M., Kodama T., Penman M., Krieger M. (1990) Coiled-coil fibrous domains mediate ligand binding by macrophage scavenger receptor type II. Nature. **343**: 570.

87. Elomaa O., Kangas M., Sahlberg C., Tuukkanen J., Sormunen R., Liakka A., Thesleff I., Kraal G., Tryggvason K. (1995) Cloning of a novel bacteria-binding receptor structurally related to scavenger receptors and expressed in a subset of macrophages. Cell. **80**: 603.

88. Kraal G., van der Laan L.J., Elomaa O., Tryggvason K. (2000) The macrophage receptor MARCO. Microbes and Infection. **2**: 313.

89. Endemann G., Stanton L.W., Madden K.S., Bryant C.M., White R.T., Protter A.A. (1993) CD36 is a receptor for oxidized low density lipoprotein. Journal of Biological Chemistry. **268**: 11811.

90. Vega M.A., Segui-Real B., Garcia J.A., Cales C., Rodriguez F., Vanderkerckhove J., Sandoval I.V. (1991) Cloning, sequencing, and expression of a cDNA encoding rat LIMP II, a novel 74-kDa lysosomal membrane protein related to the surface adhesion protein CD36. Journal of Biological Chemistry. **266**: 16818.

91. Hart K., Wilcox M. (1993) A *Drosophila* gene encoding an epithelial membrane protein with homology to CD36/LIMP II. Journal of Molecular Biology. **234**: 249.

92. Rigotti A., Acton S.L., Krieger M. (1995) The class B scavenger receptors SR-BI and CD36 are receptors for anionic phospholipids. Journal of Biological Chemistry. **270**: 16221.

93. Acton S., Rigotti A., Landschulz K.T., Xu S., Hobbs H.H., Krieger M. (1996) Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. Science. **271**: 518.

94. Greenwalt D.E., Lipsky R.H., Ockenhouse C.F., Ikeda H., Tandon N.N., Jamieson G.A. (1992) Membrane glycoprotein CD36: a review of its roles in adherence, signal transduction, and transfusion medicine. Blood. **80**: 1105.

95. Nicholson A.C., Frieda S., Pearce A., Silverstein R.L. (1995) Oxidized LDL binds to CD36 on human monocyte-derived macrophages and transfected cell

lines. Evidence implicating the lipid moiety of the lipoprotein as the binding site. Arteriosclerosis, Thrombosis, and Vascular Biology. **15**: 269.

96. Kobzik L. (1995) Lung macrophage uptake of unopsonized environmental particulates. Role of scavenger-type receptors. Journal of Immunology. **155**: 367.
97. Wright S.D., Ramos R.A., Tobias P.S., Ulevitch R.J., Mathison J.C. (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science. **249**: 131.
98. Ulevitch R.J., Tobias P.S. (1995) Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. Annual Review of Immunology. **13**: 437.
99. Wright S.D. (1995) CD14 and innate recognition of bacteria. Journal of Immunology. **155**: 6.
100. Haziot A., Ferrero E., Kontgen F., Hijiya N., Yamamoto S., Silver J., Stewart C.L., Goyert S.M. (1996) Resistance to endotoxin shock and reduced dissemination of gram- negative bacteria in CD14-deficient mice. Immunity. **4**: 407.
101. Kitchens R.L. (2000) Role of CD14 in cellular recognition of bacterial lipopolysaccharides. Chemical Immunology **74**: 61.
102. Goyert S.M., Ferrero E., Rettig W.J., Yenamandra A.K., Obata F., Le Beau M.M. (1988) The CD14 monocyte differentiation antigen maps to a region encoding growth factors and receptors. Science. **239**: 497.
103. Bazil V., Baudys M., Hilgert I., Stefanova I., Low M.G., Zbrozek J., Horejsi V. (1989) Structural relationship between the soluble and membrane-bound forms of human monocyte surface glycoprotein CD14. Molecular Immunolology. **26**: 657.
104. Tobias P.S., Mathison J., Mintz D., Lee J.D., Kravchenko V., Kato K., Pugin J., Ulevitch R.J. (1992) Participation of lipopolysaccharide-binding protein in lipopolysaccharide-dependent macrophage activation. American Journal of Respiratory Cell and Molecular Biology, **7**: 239.
105. Tobias P.S., Ulevitch R.J. (1993) Lipopolysaccharide binding protein and CD14 in LPS dependent macrophage activation. Immunobiology. **187**: 227.
106. Lee J.D., Kato K., Tobias P.S., Kirkland T.N., Ulevitch R.J. (1992) Transfection of CD14 into 70Z/3 cells dramatically enhances the sensitivity to complexes of lipopolysaccharide (LPS) and LPS binding protein. Journal of Experimental Medicine. **175**: 1697.
107. Golenbock D.T., Liu Y., Millham F.H., Freeman M.W., Zoeller R.A. (1993) Surface expression of human CD14 in Chinese hamster ovary fibroblasts imparts macrophage-like responsiveness to bacterial endotoxin. Journal of Biological Chemistry. **268**: 22055.
108. Delude R.L., Savedra R.J., Zhao H., Thieringer R., Yamamoto S., Fenton M.J., Golenbock D.T. (1995) CD14 enhances cellular responses to endotoxin without imparting ligand- specific recognition. Proceedings of the National Academy of Sciences of the United States of America. **92**: 9288.
109. Nishijima M., Hara-Kuge S., Takasuka N., Akagawa K., Setouchi M., Matsuura K., Yamamoto S., Akamatsu Y. (1994) Identification of a biochemical lesion, and characteristic response to lipopolysaccharide (LPS) of

a cultured macrophage-like cell mutant with defective LPS-binding. Journal of Biochemistry (Tokyo). **116**: 1082.

110. Haziot A., Rong G.W., Silver J., Goyert S.M. (1993) Recombinant soluble CD14 mediates the activation of endothelial cells by lipopolysaccharide. Journal of Immunology. **151**: 1500.
111. Frey E.A., Miller D.S., Jahr T.G., Sundan A., Bazil V., Espevik T., Finlay B.B., Wright S.D. (1992) Soluble CD14 participates in the response of cells to lipopolysaccharide. Journal of Experimental Medicine. **176**: 1665.
112. Pugin J., Schurer-Maly C.C., Leturcq D., Moriarty A., Ulevitch R.J., Tobias P.S. (1993) Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14. Proceedings of the National Academy of Sciences of the United States of America. **90**: 2744.
113. Tapping R.I., Tobias P.S. (1997) Cellular binding of soluble CD14 requires lipopolysaccharide (LPS) and LPS-binding protein. Journal of Biological Chemistry. **272**: 23157.
114. Ulevitch R.J., Tobias P.S. (1999) Recognition of gram-negative bacteria and endotoxin by the innate immune system. Current Opinion in Immunology **11**: 19.
115. Diks S.H., van Deventer S.J., Peppelenbosch M.P. (2001) Lipopolysaccharide recognition, internalisation, signalling and other cellular effects. Journal of Endotoxin Research. **7**: 335.
116. Anderson K.V., Bokla L., Nusslein-Volhard C. (1985) Establishment of dorsal-ventral polarity in the *Drosophila* embryo: the induction of polarity by the Toll gene product. Cell. **42**: 791-8.
117. Sun H., Bristow B.N., Qu G., Wasserman S.A. (2002) A heterotrimeric death domain complex in Toll signaling. Proceedings of the National Academy of Sciences of the United States of America. **99**: 12871-6.
118. Akira S. (2003) Mammalian Toll-like receptors. Current Opinion in Immunology. **15**: 5-11.
119. Akira S., Hoshino K., Kaisho T. (2000) The role of Toll-like receptors and MyD88 in innate immune responses. Journal of Endotoxin Research. **6**: 383.
120. Fitzgerald K.A., Palsson-McDermott E.M., Bowie A.G., Jefferies C.A., Mansell A.S., Brady G., Brint E., Dunne A., Gray P., Harte M.T., McMurray D., Smith D.E., Sims J.E., Bird T.A., O'Neill L.A. (2001) Mal (MyD88-adapter-like) is required for Toll-like receptor-4 signal transduction. Nature. **413**: 78.
121. Kaisho T., Akira S. (2001) Dendritic-cell function in Toll-like receptor- and MyD88-knockout mice. Trends in Immunology. **22**: 78.
122. Kawai T., Adachi O., Ogawa T., Takeda K., Akira S. (1999) Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity. **11**: 115.
123. Medzhitov R., Preston-Hurlburt P., Kopp E., Stadlen A., Chen C., Ghosh S., Janeway C.A., Jr. (1998) MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. Molecular Cell. **2**: 253.

124. Schnare M., Holt A.C., Takeda K., Akira S., Medzhitov R. (2000) Recognition of CpG DNA is mediated by signaling pathways dependent on the adaptor protein MyD88. Current Biology. **10**: 1139.
125. Takeuchi O., Hoshino K., Akira S. (2000) Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. Journal of Immunology. **165**: 5392.
126. Takeuchi O., Takeda K., Hoshino K., Adachi O., Ogawa T., Akira S. (2000) Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. International Immunology. **12**: 113.
127. Wang Q., Dziarski R., Kirschning C.J., Muzio M., Gupta D. (2001) Micrococci and peptidoglycan activate TLR2-->MyD88-->IRAK-->TRAF-->NIK-- >IKK-->NF-kappaB signal transduction pathway that induces transcription of interleukin-8. Infection and Immunity. **69**: 2270.
128. Beutler B. (2000) Endotoxin, Toll-like receptor 4, and the afferent limb of innate immunity. Current Opinion in Microbiology. **3**: 23.
129. Beutler B. (2000) Tlr4: central component of the sole mammalian LPS sensor. Current Opinion in Immunology. **12**: 20.
130. Poltorak A., He X., Smirnova I., Liu M.Y., Huffel C.V., Du X., Birdwell D., Alejos E., Silva M., Galanos C., Freudenberg M., Ricciardi-Castagnoli P., Layton B., Beutler B. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science. **282**: 2085.
131. Akashi S., Shimazu R., Ogata H., Nagai Y., Takeda K., Kimoto M., Miyake K. (2000) Cutting edge: cell surface expression and lipopolysaccharide signaling via the Toll-like receptor 4-MD-2 complex on mouse peritoneal macrophages. Journal of Immunology. **164**: 3471.
132. Dziarski R., Wang Q., Miyake K., Kirschning C.J., Gupta D. (2001) MD-2 enables Toll-like receptor 2 (TLR2)-mediated responses to lipopolysaccharide and enhances TLR2-mediated responses to Gram- positive and Gram-negative bacteria and their cell wall components. Journal of Immunology. **166**: 1938.
133. Schromm A.B., Lien E., Henneke P., Chow J.C., Yoshimura A., Heine H., Latz E., Monks B.G., Schwartz D.A., Miyake K., Golenbock D.T. (2001) Molecular genetic analysis of an endotoxin nonresponder mutant cell line: a point mutation in a conserved region of MD-2 abolishes endotoxin-induced signaling. Journal of Experimental Medicine. **194**: 79.
134. Shimazu R., Akashi S., Ogata H., Nagai Y., Fukudome K., Miyake K., Kimoto M. (1999) MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll- like receptor 4. Journal of Experimental Medicine. **189**: 1777.
135. Viriyakosol S., Tobias P.S., Kitchens R.L., Kirkland T.N. (2001) MD-2 Binds to Bacterial Lipopolysaccharide. Journal of Biological Chemistry. **276**: 38044.
136. Eaves-Pyles T., Murthy K., Liaudet L., Virag L., Ross G., Soriano F.G., Szabo C., Salzman A.L. (2001) Flagellin, a novel mediator of *Salmonella*-induced epithelial activation and systemic inflammation: I kappa B alpha degradation, induction of nitric oxide synthase, induction of proinflammatory mediators, and cardiovascular dysfunction. Journal of Immunology. **166**: 1248.

137. Brightbill H.D., Libraty D.H., Krutzik S.R., Yang R.B., Belisle J.T., Bleharski J.R., Maitland M., Norgard M.V., Plevy S.E., Smale S.T., Brennan P.J., Bloom B.R., Godowski P.J., Modlin R.L. (1999) Host defense mechanisms triggered by microbial lipoproteins through Toll-like receptors. Science. **285**: 732.

138. Aliprantis A.O., Yang R.B., Mark M.R., Suggett S., Devaux B., Radolf J.D., Klimpel G.R., Godowski P., Zychlinsky A. (1999) Cell activation and apoptosis by bacterial lipoproteins through Toll- like receptor-2. Science. **285**: 736.

139. Tachado S.D., Gerold P., McConville M.J., Baldwin T., Quilici D., Schwarz R.T., Schofield L. (1996) Glycosylphosphatidylinositol toxin of *Plasmodium* induces nitric oxide synthase expression in macrophages and vascular endothelial cells by a protein tyrosine kinase-dependent and protein kinase C-dependent signaling pathway. Journal of Immunology. **156**: 1897.

140. Campos M.A., Almeida I.C., Takeuchi O., Akira S., Valente E.P., Procopio D.O., Travassos L.R., Smith J.A., Golenbock D.T., Gazzinelli R.T. (2001) Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. Journal of Immunology. **167**: 416.

141. Ropert C., Gazzinelli R.T. (2000) Signaling of immune system cells by glycosylphosphatidylinositol (GPI) anchor and related structures derived from parasitic protozoa. Current Opinion Microbiology. **3**: 395.

142. Wetzler L.M. (2003) The role of Toll-like receptor 2 in microbial disease and immunity. Vaccine. **21 Suppl**: S55-S60.

143. Gewirtz A.T., Navas T.A., Lyons S., Godowski P.J., Madara J.L. (2001) Cutting edge: bacterial flagellin activates basolaterally expressed tlr5 to induce epithelial proinflammatory gene expression. Journal of Immunology. **167**: 1882.

144. Hayashi F., Smith K.D., Ozinsky A., Hawn T.R., Yi E.C., Goodlett D.R., Eng J.K., Akira S., Underhill D.M., Aderem A. (2001) The innate immune response to bacterial flagellin is mediated by Toll- like receptor 5. Nature. **410**: 1099.

145. Zhu F.G., Pisetsky D.S. (2001) Role of the heat shock protein 90 in immune response stimulation by bacterial DNA and synthetic oligonucleotides. Infection and Immunity. **69**: 5546.

146. Alexopoulou L., Holt A.C., Medzhitov R., Flavell R.A. (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature. **413**: 732.

147. Edwards A.D., Diebold S.S., Slack E.M., Tomizawa H., Hemmi H., Kaisho T., Akira S., Reis e Sousa C. (2003) Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines. European Journal of Immunology. **33**: 827.

148. Lee J., Chuang T.H., Redecke V., She L., Pitha P.M., Carson D.A., Raz E., Cottam H.B. (2003) Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: Activation of Toll-like receptor 7. Proceedings of the National Academy of Sciences of the United States of America. **(in press)**.

149. Lemaitre B., Reichhart J.M., Hoffmann J.A. (1997) *Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. Proceedings of the National Academy of Sciences of the United States of America. **94**: 14614.

150. Beg A.A. (2002) Endogenous ligands of Toll-like receptors: implications for regulating inflammatory and immune responses. Trends in Immunology. **23**: 509.

151. Johnson G.B., Brunn G.J., Tang A.H., Platt J.L. (2003) Evolutionary clues to the functions of the Toll-like family as surveillance receptors. Trends in Immunology. **24**: 19.

152. Guillot L., Balloy V., McCormack F.X., Golenbock D.T., Chignard M., Si-Tahar M. (2002) Cutting edge: the immunostimulatory activity of the lung surfactant protein-A involves Toll-like receptor 4. Journal of Immunology. **168**: 5989.

153. Ohashi K., Burkart V., Flohe S., Kolb H. (2000) Cutting edge: heat shock protein 60 is a putative endogenous ligand of the Toll-like receptor-4 complex. Journal of Immunology. **164**: 558.

154. Asea A., Rehli M., Kabingu E., Boch J.A., Bare O., Auron P.E., Stevenson M.A., Calderwood S.K. (2002) Novel signal transduction pathway utilized by extracellular HSP70: role of Toll-like receptor (TLR) 2 and TLR4. Journal of Biological Chemistry. **277**: 15028.

155. Termeer C., Benedix F., Sleeman J., Fieber C., Voith U., Ahrens T., Miyake K., Freudenberg M., Galanos C., Simon J.C. (2002) Oligosaccharides of hyaluronan activate dendritic cells vial Toll-like receptor 4. Journal of Experimental Medicine. **195**: 99.

156. Johnson G.B., Brunn G.J., Kodaira Y., Platt J.L. (2002) Receptor-mediated monitoring of tissue well-being via detection of soluble heparan sulfate by Toll-like receptor 4. Journal of Immunology. **168**: 5233.

157. Mansell A.S., Reinicke A., Worrall D.M., O'Neill L.A.J. (2001) The serine protease inhibitor antithrombin III inhibits LPS-mediated NF-kappa beta activation by TLR-4. FEBS Letters. **508**: 313.

158. Aparicio S., Chapman J., Stupka E., Putnam N., Chia J.M., Dehal P.e.a. (2002) Whole-genome shotgun assembly and analysis of the genome of *Fugu rubripes*. Science. **297**: 1301.

159. Oshiumi H., Tsujita T., Shida K., Matsumoto M., Ikeo K., Seya T. (2003) Prediction of the prototype of the human Toll-like receptor gene family from the pufferfish, *Fugu rubripes*, genome. Immunogenetics. **54**: 791.

160. Stafford J.L., Ellestad K.K., Magor K.E., Belosevic M., Magor B.G. (2003) A Toll-like receptor (TLR) gene that is up-regulated in activated goldfish macrophages. Developmental and Comparative Immunology. **27**: 685.

161. Borelli V., Banfi E., Perrotta M.G., Zabucchi G. (1999) Myeloperoxidase exerts microbicidal activity against Mycobacterium tuberculosis. Infection and Immunity. **67**: 4149.

162. Gabay J.E., Almeida R.P. (1993) Antibiotic peptides and serine protease homologs in human polymorphonuclear leukocytes: defensins and azurocidin. Current Opinion in Immunology. **5**: 97.

163. Ganz T., Lehrer R.I. (1997) Antimicrobial peptides of leukocytes. Current Opinion in Hematology. **4**: 53.

164. Levy O., Weiss J., Zarember K., Ooi C.E., Elsbach P. (1993) Antibacterial 15-kDa protein isoforms (p15s) are members of a novel family of leukocyte proteins. Journal of Biological Chemistry. **268**: 6058.

165. Levy O., Ooi C.E., Elsbach P., Doerfler M.E., Lehrer R.I., Weiss J. (1995) Antibacterial proteins of granulocytes differ in interaction with endotoxin. Comparison of bactericidal/permeability-increasing protein, p15s, and defensins. Journal of Immunology. **154**: 5403.

166. Lichtenstein A.K., Ganz T., Selsted M.E., Lehrer R.I. (1988) Synergistic cytolysis mediated by hydrogen peroxide combined with peptide defensins. Cellular Immunolology. **114**: 104.

167. Lee G.R. (1983) The anemia of chronic disease. Seminars in Hematology. **20**: 61.

168. Kent S., Weinberg E.D., Stuart-Macadam P. (1994) The etiology of the anemia of chronic disease and infection. Journal of Clinical Epidemiology. **47**: 23.

169. Weinberg E.D. (1984) Iron withholding: a defense against infection and neoplasia. Physiological Reviews. **64**: 65.

170. Weiss G., Fuchs D., Hausen A., Reibnegger G., Werner E.R., Werner-Felmayer G., Wachter H. (1992) Iron modulates interferon-gamma effects in the human myelomonocytic cell line THP-1. Experimental Hematology. **20**: 305.

171. Jacobs A. (1977) Low molecular weight intracellular iron transport compounds. Blood. **50**: 433.

172. Nunez M.T., Gaete V., Watkins J.A., Glass J. (1990) Mobilization of iron from endocytic vesicles. The effects of acidification and reduction. Journal of Biological Chemistry. **265**: 6688.

173. Byrd T.F., Horwitz M.A. (1991) Lactoferrin inhibits or promotes *Legionella pneumophila* intracellular multiplication in nonactivated and interferon gamma-activated human monocytes depending upon its degree of iron saturation. Iron- lactoferrin and nonphysiologic iron chelates reverse monocyte activation against *Legionella pneumophila*. Journal of Clinical Investigation. **88**: 1103.

174. Reif D.W., Simmons R.D. (1990) Nitric oxide mediates iron release from ferritin. Archives of Biochemistry and Biophysics. **283**: 537.

175. Drapier J.C., Hirling H., Wietzerbin J., Kaldy P., Kuhn L.C. (1993) Biosynthesis of nitric oxide activates iron regulatory factor in macrophages. EMBO Journal. **12**: 3643.

176. Weiss G., Werner-Felmayer G., Werner E.R., Grunewald K., Wachter H., Hentze M.W. (1994) Iron regulates nitric oxide synthase activity by controlling nuclear transcription. Journal of Experimental Medicine. **180**: 969.

177. Weiss G., Kastner S., Brock J., Thaler J., Grunewald K. (1997) Modulation of transferrin receptor expression by dexrazoxane (ICRF-187) via activation of iron regulatory protein. Biochemical Pharmacology. **53**: 1419.

178. Weiss G., Bogdan C., Hentze M.W. (1997) Pathways for the regulation of macrophage iron metabolism by the anti- inflammatory cytokines IL-4 and IL-13. Journal of Immunology. **158**: 420.

179. Masson P.L., Heremans J.F., Schonne E. (1969) Lactoferrin, an iron-binding protein in neutrophilic leukocytes. Journal of Experimental Medicine. **130**: 643.

180. Ambruso D.R., Johnston R.B., Jr. (1981) Lactoferrin enhances hydroxyl radical production by human neutrophils, neutrophil particulate fractions, and an enzymatic generating system. Journal of Clinical Investigation. **67**: 352.

181. Djeha A., Perez-Arellano J.L., Hayes S.L., Oria R., Simpson R.J., Raja K.B., Brock J.H. (1995) Cytokine-mediated regulation of transferrin synthesis in mouse macrophages and human T lymphocytes. Blood. **85**: 1036.

182. Byrd T.F., Horwitz M.A. (1989) Interferon gamma-activated human monocytes downregulate transferrin receptors and inhibit the intracellular multiplication of Legionella pneumophila by limiting the availability of iron. Journal of Clinical Investigation. **83**: 1457.

183. Byrd T.F., Horwitz M.A. (2000) Aberrantly low transferrin receptor expression on human monocytes is associated with nonpermissiveness for *Legionella pneumophila* growth. Journal of Infectious Diseases. **181**: 1394.

184. Hamilton T.A., Gray P.W., Adams D.O. (1984) Expression of the transferrin receptor on murine peritoneal macrophages is modulated by *in vitro* treatment with interferon gamma. Cellular Immunology. **89**: 478.

185. Hamilton T.A., Weiel J.E., Adams D.O. (1984) Expression of the transferrin receptor in murine peritoneal macrophages is modulated in the different stages of activation. Journal of Immunological Methods. **132**: 2285-90.

186. Hirata T., Bitterman P.B., Mornex J.-F., Crystal R.G. (1986) Expression of the transferrin receptor gene during the process of mononuclear phagocyte maturation. Journal of Immunological Methods. **4**: 1339-45.

187. Jiang X., Baldwin C.L. (1993) Iron augments macrophage-mediated killing of *Brucella abortus* alone and in conjunction with interferon-gamma. Cellular Immunology. **148**: 397.

188. Weiss G., Goossen B., Doppler W., Fuchs D., Pantopoulos K., Werner-Felmayer G., Wachter H., Hentze M.W. (1993) Translational regulation via iron-responsive elements by the nitric oxide/NO-synthase pathway. EMBO Journal. **12**: 3651.

189. Weiss G., Murr C., Zoller H., Haun M., Widner B., Ludescher C., Fuchs D. (1999) Modulation of neopterin formation and tryptophan degradation by Th1- and Th2-derived cytokines in human monocytic cells. Clinical and Experimental Immunology. **116**: 435.

190. Forbes J.R., Gros P. (2001) Divalent-metal transport by NRAMP proteins at the interface of host- pathogen interactions. Trends in Microbiology. **9**: 397.

191. Cellier M.F., Bergevin I., Boyer E., Richer E. (2001) Polyphyletic origins of bacterial Nramp transporters. Trends in Genetics. **17**: 365.

192. Blackwell J.M., Barton C.H., White J.K., Roach T.I., Shaw M.A., Whitehead S.H., Mock B.A., Searle S., Williams H., Baker A.M. (1994) Genetic

regulation of leishmanial and mycobacterial infections: the Lsh/Ity/Bcg gene story continues. Immunology Letters. **43**: 99.

193. Govoni G., Vidal S., Gauthier S., Skamene E., Malo D., Gros P. (1996) The Bcg/Ity/Lsh locus: genetic transfer of resistance to infections in C57BL/6J mice transgenic for the Nramp1 Gly169 allele. Infection and Immunity. **64**: 2923.
194. Vidal S., Tremblay M.L., Govoni G., Gauthier S., Sebastiani G., Malo D., Skamene E., Olivier M., Jothy S., Gros P. (1995) The Ity/Lsh/Bcg locus: natural resistance to infection with intracellular parasites is abrogated by disruption of the Nramp1 gene. Journal of Experimental Medicine. **182**: 655.
195. Vidal S., Gros P., Skamene E. (1995) Natural resistance to infection with intracellular parasites: molecular genetics identifies Nramp1 as the Bcg/Ity/Lsh locus. Journal of Leukocyte Biology. **58**: 382.
196. Vidal S.M., Malo D., Vogan K., Skamene E., Gros P. (1993) Natural resistance to infection with intracellular parasites: isolation of a candidate for Bcg. Cell. **73**: 769.
197. Gruenheid S., Cellier M., Vidal S., Gros P. (1995) Identification and characterization of a second mouse Nramp gene. Genomics. **25**: 514.
198. Vidal S.M., Pinner E., Lepage P., Gauthier S., Gros P. (1996) Natural resistance to intracellular infections: Nramp1 encodes a membrane phosphoglycoprotein absent in macrophages from susceptible (Nramp1 D169) mouse strains. Journal of Immunology. **157**: 3559.
199. Malo D., Vogan K., Vidal S., Hu J., Cellier M., Schurr E., Fuks A., Bumstead N., Morgan K., Gros P. (1994) Haplotype mapping and sequence analysis of the mouse Nramp gene predict susceptibility to infection with intracellular parasites. Genomics. **23**: 51.
200. Hackam D.J., Rotstein O.D., Zhang W., Gruenheid S., Gros P., Grinstein S. (1998) Host resistance to intracellular infection: mutation of natural resistance-associated macrophage protein 1 (Nramp1) impairs phagosomal acidification. Journal of Experimental Medicine. **188**: 351.
201. Gruenheid S., Canonne-Hergaux F., Gauthier S., Hackam D.J., Grinstein S., Gros P. (1999) The iron transport protein NRAMP2 is an integral membrane glycoprotein that colocalizes with transferrin in recycling endosomes. Journal of Experimental Medicine. **189**: 831.
202. Govoni G., Vidal S., Cellier M., Lepage P., Malo D., Gros P. (1995) Genomic structure, promoter sequence, and induction of expression of the mouse Nramp1 gene in macrophages. Genomics. **27**: 9.
203. Gruenheid S., Pinner E., Desjardins M., Gros P. (1997) Natural resistance to infection with intracellular pathogens: the Nramp1 protein is recruited to the membrane of the phagosome. Journal of Experimental Medicine. **185**: 717.
204. Jabado N., Jankowski A., Dougaparsad S., Picard V., Grinstein S., Gros P. (2000) Natural resistance to intracellular infections: natural resistance-associated macrophage protein 1 (Nramp1) functions as a pH-dependent manganese transporter at the phagosomal membrane. Journal of Experimental Medicine. **192**: 1237.

205. Atkinson P.G., Barton C.H. (1999) High level expression of Nramp1G169 in RAW264.7 cell transfectants: analysis of intracellular iron transport. Immunology. **96**: 656.

206. Barton C.H., Biggs T.E., Baker S.T., Bowen H., Atkinson P.G. (1999) Nramp1: a link between intracellular iron transport and innate resistance to intracellular pathogens. Journal of Leukocyte Biology. **66**: 757.

207. Lee P.L., Gelbart T., West C., Halloran C., Beutler E. (1998) The human Nramp2 gene: characterization of the gene structure, alternative splicing, promoter region and polymorphisms. Blood Cells Molecules and Diseases. **24**: 199.

208. Picard V., Govoni G., Jabado N., Gros P. (2000) Nramp 2 (DCT1/DMT1) expressed at the plasma membrane transports iron and other divalent cations into a calcein-accessible cytoplasmic pool. Journal of Biological Chemistry. **275**: 35738.

209. Wardrop S.L., Richardson D.R. (2000) Interferon-gamma and lipopolysaccharide regulate the expression of Nramp2 and increase the uptake of iron from low relative molecular mass complexes by macrophages. European Journal of Biochemistry. **267**: 6586.

210. Dorschner M.O., Philips R.B. (1999) Comparative analysis of two NRAMP loci from rainbow trout. DNA and Cell Biology. **18**: 573.

211. Blackwell J.M., Goswami T., Evans C.A., Sibthorpe D., Papo N., White J.K., Searle S., Miller E.N., Peacock C.S., Mohammed H., Ibrahim M.A. (2001) SLC11A1 (formerly NRAMP1) and disease resistance. Cellular Microbiology. **3**: 773.

212. Chen H., Waldbieser G.C., Rice C.D., Elibol B., Wolter W.R., Hanson L.A. (2002) Isolation and characterization of channel catfish natural resistance associated macrophage protein gene. Developmental and Comparative Immunology. **26**: 517.

213. Overath P., Haag J., Mameza M.G., Lischke A. (1999) Freshwater fish trypanosomes: definition of two types, host control by antibodies and lack of antigenic variation. Parasitology. **119**: 591.

214. Saeij J.P., Wiegertjes G.F. (1999) Identification and characterization of a fish natural resistance-associated macrophage protein (NRAMP) cDNA. Immunogenetics. **50**: 60.

215. Ozaki Y., Edelstein M.P., Duch D.S. (1987) The actions of interferon and antiinflammatory agents of induction of indoleamine 2,3-dioxygenase in human peripheral blood monocytes. Biochemical and Biophysical Research Communications. **144**: 1147.

216. Taylor M.W., Feng G.S. (1991) Relationship between interferon-gamma, indoleamine 2,3-dioxygenase, and tryptophan catabolism. FASEB Journal. **5**: 2516.

217. Hayaishi O., Hirata F., Ohnishi T., Henry J.P., Rosenthal I., Katoh A. (1977) Indoleamine 2,3-dioxygenase: incorporation of 18O2-- and 18O2 into the reaction products. Journal of Biological Chemistry. **252**: 3548.

218. Tone S., Takikawa O., Habara-Ohkubo A., Kadoya A., Yoshida R., Kido R. (1990) Primary structure of human indoleamine 2,3-dioxygenase deduced from the nucleotide sequence of its cDNA. Nucleic Acids Research. **18**: 367.
219. Pfefferkorn E.R. (1984) Interferon gamma blocks the growth of *Toxoplasma gondii* in human fibroblasts by inducing the host cells to degrade tryptophan. Proceedings of the National Academy of Sciences of the United States of America. **81**: 908.
220. Byrne G.I., Lehmann L.K., Landry G.J. (1986) Induction of tryptophan catabolism is the mechanism for gamma- interferon-mediated inhibition of intracellular *Chlamydia psittaci* replication in T24 cells. Infection and Immunity. **53**: 347.
221. Carlin J.M., Borden E.C., Sondel P.M., Byrne G.I. (1989) Interferon-induced indoleamine 2,3-dioxygenase activity in human mononuclear phagocytes. Journal of Leukocyte Biology. **45**: 29.
222. Schmitz J.L., Carlin J.M., Borden E.C., Byrne G.I. (1989) Beta interferon inhibits *Toxoplasma gondii* growth in human monocyte- derived macrophages. Infection and Immunity. **57**: 3254.
223. Murray H.W., Szuro-Sudol A., Wellner D., Oca M.J., Granger A.M., Libby D.M., Rothermel C.D., Rubin B.Y. (1989) Role of tryptophan degradation in respiratory burst-independent antimicrobial activity of gamma interferon-stimulated human macrophages. Infection and Immunity. **57**: 845.
224. Paguirigan A.M., Byrne G.I., Becht S., Carlin J.M. (1994) Cytokine-mediated indoleamine 2,3-dioxygenase induction in response to *Chlamydi*a infection in human macrophage cultures. Infection and Immunity. **62**: 1131.
225. Daubener W., MacKenzie C.R. (1999) IFN-gamma activated indoleamine 2,3-dioxygenase activity in human cells is an antiparasitic and an antibacterial effector mechanism. Advances in Experimental Medicine and Biology. **467**: 517.
226. MacKenzie C.R., Hadding U., Daubener W. (1998) Interferon-gamma-induced activation of indoleamine 2,3-dioxygenase in cord blood monocyte-derived macrophages inhibits the growth of group B *streptococci*. Journal of Infectious Diseases. **178**: 875.
227. MacKenzie C.R., Langen R., Takikawa O., Daubener W. (1999) Inhibition of indoleamine 2,3-dioxygenase in human macrophages inhibits interferon-gamma-induced bacteriostasis but does not abrogate toxoplasmastasis. European Journal of Immunology. **29**: 3254.
228. Ozaki Y., Borden E.C., Smalley R.V., Brown R.R. (1991) Interferon type I and II antagonism: a novel regulatory mechanism of indoleamine dioxygenase induction in human peripheral blood monocytes and peritoneal macrophages. Advances in Experimental Medicine and Biology. **294**: 547.
229. Werner-Felmayer G., Werner E.R., Fuchs D., Hausen A., Reibnegger G., Wachter H. (1989) Characteristics of interferon induced tryptophan metabolism in human cells *in vitro*. Biochimica et Biophysica Acta. **1012**: 140.
230. Werner-Felmayer G., Werner E.R., Fuchs D., Hausen A., Reibnegger G., Wachter H. (1990) Neopterin formation and tryptophan degradation by a

human myelomonocytic cell line (THP-1) upon cytokine treatment. Cancer Research. **50**: 2863.

231. Werner-Felmayer G., Werner E.R., Fuchs D., Hausen A., Reibnegger G., Wachter H. (1989) Tumour necrosis factor-alpha and lipopolysaccharide enhance interferon- induced tryptophan degradation and pteridine synthesis in human cells. Biological Chemistry Hoppe-Seyler. **370**: 1063.
232. Melillo G., Cox G.W., Radzioch D., Varesio L. (1993) Picolinic acid, a catabolite of L-tryptophan, is a costimulus for the induction of reactive nitrogen intermediate production in murine macrophages. Journal of Immunology. **150**: 4031.
233. Melillo G., Cox G.W., Biragyn A., Sheffler L.A., Varesio L. (1994) Regulation of nitric-oxide synthase mRNA expression by interferon-gamma and picolinic acid. Journal of Biological Chemistry. **269**: 8128.
234. Melillo G., Bosco M.C., Musso T., Varesio L. (1996) Immunobiology of picolinic acid. Advances in Experimental Medicine and Biology. **398**: 135.
235. Varesio L., Clayton M., Blasi E., Ruffman R., Radzioch D. (1990) Picolinic acid, a catabolite of tryptophan, as the second signal in the activation of IFN-gamma-primed macrophages. Journal of Immunology. **145**: 4265.
236. Melillo G., Musso T., Sica A., Taylor L.S., Cox G.W., Varesio L. (1995) A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. Journal of Experimental Medicine. **182**: 1683.
237. Bosco M.C., Rapisarda A., Massazza S., Melillo G., Young H., Varesio L. (2000) The tryptophan catabolite picolinic acid selectively induces the chemokines macrophage inflammatory protein-1 alpha and -1 beta in macrophages. Journal of Immunology. **164**: 3283.
238. Taub D.D., Conlon K., Lloyd A.R., Oppenheim J.J., Kelvin D.J. (1993) Preferential migration of activated CD4+ and CD8+ T cells in response to MIP-1 alpha and MIP-1 beta. Science. **260**: 355.
239. Taub D.D., Lloyd A.R., Wang J.M., Oppenheim J.J., Kelvin D.J. (1993) The effects of human recombinant MIP-1 alpha, MIP-1 beta, and RANTES on the chemotaxis and adhesion of T cell subsets. Advances in Experimantal Medicine and Biology. **351**: 139.
240. Iyer G.Y.N., Islam M.F., Quastel J.H. (1961) Biochemical aspects of phagocytosis. Nature. **192**: 535.
241. Paul B., Sbarra A.J. (1968) The role of the phagocyte in host-parasite interactions. 13. The direct quantitative estimation of H2O2 in phagocytizing cells. Biochemistry and Biophysics Acta. **156**: 168-78.
242. Dinauer M.C. (1993) The respiratory burst oxidase and the molecular genetics of chronic granulomatous disease. Critical Reviews in Clinical Laboratory Sciences. **30**: 329.
243. Segal A.W. (1996) The NADPH oxidase and chronic granulomatous disease. Molecular Medicine Today. **2**: 129.
244. Briggs R.T., Drath D.B., Karnovsky M.L., Karnovsky M.J. (1975) Localization of NADH oxidase on the surface of human polymorphonuclear

leukocytes by a new cytochemical method. Journal of Cellular Biology. **67**: 566.

245. Parkos C.A., Allen R.A., Cochrane C.G., Jesaitis A.J. (1987) Purified cytochrome b from human granulocyte plasma membrane is comprised of two polypeptides with relative molecular weights of 91,000 and 22,000. Journal of Clinical Investigation. **80**: 732.
246. Parkos C.A., Allen R.A., Cochrane C.G., Jesaitis A.J. (1988) The quaternary structure of the plasma membrane b-type cytochrome of human granulocytes. Biochemistry and Biophysics Acta. **932**: 71.
247. Segal A.W. (1987) Absence of both cytochrome b-245 subunits from neutrophils in X-linked chronic granulomatous disease. Nature. **326**: 88.
248. Nunoi H., Rotrosen D., Gallin J.I., Malech H.L. (1988) Two forms of autosomal chronic granulomatous disease lack distinct neutrophil cytosol factors. Science. **242**: 1298.
249. Tsunawaki S., Mizunari H., Nagata M., Tatsuzawa O., Kuratsuji T. (1994) A novel cytosolic component, p40phox, of respiratory burst oxidase associates with p67phox and is absent in patients with chronic granulomatous disease who lack p67phox. Biochemical and Biophysical Research Communications. **199**: 1378.
250. Tsunawaki S., Yoshikawa K. (2000) Relationships of p40(phox) with p67(phox) in the activation and expression of the human respiratory burst NADPH oxidase. Journal of Biochemistry. (Tokyo). **128**: 777.
251. Volpp B.D., Nauseef W.M., Clark R.A. (1988) Two cytosolic neutrophil oxidase components absent in autosomal chronic granulomatous disease. Science. **242**: 1295.
252. Park J.W., Ma M., Ruedi J.M., Smith R.M., Babior B.M. (1992) The cytosolic components of the respiratory burst oxidase exist as a M(r) approximately 240,000 complex that acquires a membrane-binding site during activation of the oxidase in a cell-free system. Journal of Biological Chemistry. **267**: 17327.
253. Park J.W., Benna J.E., Scott K.E., Christensen B.L., Chanock S.J., Babior B.M. (1994) Isolation of a complex of respiratory burst oxidase components from resting neutrophil cytoso. Biochemistry. **33**: 2907.
254. Nauseef W.M., Volpp B.D., McCormick S., Leidal K.G., Clark R.A. (1991) Assembly of the neutrophil respiratory burst oxidase. Protein kinase C promotes cytoskeletal and membrane association of cytosolic oxidase components. Journal of Biological Chemistry. **266**: 5911.
255. Park H.S., Kim I.S., Park J.W. (1999) Phosphorylation induces conformational changes in the leukocyte NADPH oxidase subunit p47(phox). Biochemical and Biophysical Research Communications. **256**: 38.
256. Park J.W., Babior B.M. (1992) The translocation of respiratory burst oxidase components from cytosol to plasma membrane is regulated by guanine nucleotides and diacylglycerol [published erratum appears in J Biol Chem 1993 Jan 15;268(2):1500],. Journal of Biological Chemistry. **267**: 19901.
257. Park J.W., Ahn S.M. (1995) Translocation of recombinant p47phox cytosolic component of the phagocyte oxidase by in vitro phosphorylation. Biochemical and Biophysical Research Communications. **211**: 410.

258. Inanami O., Johnson J.L., McAdara J.K., Benna J.E., Faust L.R., Newburger P.E., Babior B.M. (1998) Activation of the leukocyte NADPH oxidase by phorbol ester requires the phosphorylation of p47PHOX on serine 303 or 304. Journal of Biological Chemistry. **273**: 9539.

259. Finan P., Shimizu Y., Gout I., Hsuan J., Truong O., Butcher C., Bennett P., Waterfield M.D., Kellie S. (1994) An SH3 domain and proline-rich sequence mediate an interaction between two components of the phagocyte NADPH oxidase complex. Journal of Biological Chemistry. **269**: 13752.

260. Heyworth P.G., Curnutte J.T., Nauseef W.M., Volpp B.D., Pearson D.W., Rosen H., Clark R.A. (1991) Neutrophil nicotinamide adenine dinucleotide phosphate oxidase assembly. Translocation of p47-phox and p67-phox requires interaction between p47-phox and cytochrome b558. Journal of Clinical Investigation. **87**: 352.

261. Rotrosen D., Leto T.L. (1990) Phosphorylation of neutrophil 47-kDa cytosolic oxidase factor. Translocation to membrane is associated with distinct phosphorylation events. Journal of Biological Chemistry. **265**: 19910.

262. El Benna J., Faust L.P., Babior B.M. (1994) The phosphorylation of the respiratory burst oxidase component p47phox during neutrophil activation. Phosphorylation of sites recognized by protein kinase C and by proline-directed kinases. Journal of Biological Chemistry. **269**: 23431.

263. El Benna J., Park J.W., Ruedi J.M., Babior B.M. (1995) Cell-free activation of the respiratory burst oxidase by protein kinase C. Blood Cells, Molecules and Diseases. **21**: 201.

264. El Benna J., Han J., Park J.W., Schmid E., Ulevitch R.J., Babior B.M. (1996) Activation of p38 in stimulated human neutrophils: phosphorylation of the oxidase component p47phox by p38 and ERK but not by JNK. Archives of Biochemistry and Biophysics. **334**: 395.

265. El Benna J., Faust R.P., Johnson J.L., Babior B.M. (1996) Phosphorylation of the respiratory burst oxidase subunit p47phox as determined by two-dimensional phosphopeptide mapping. Phosphorylation by protein kinase C, protein kinase A, and a mitogen-activated protein kinase. Journal of Biological Chemistry. **271**: 6374.

266. Nixon J.B., McPhail L.C. (1999) Protein kinase C (PKC) isoforms translocate to Triton-insoluble fractions in stimulated human neutrophils: correlation of conventional PKC with activation of NADPH oxidase. Journal of Immunology. **163**: 4574.

267. Larsen E.C., DiGennaro J.A., Saito N., Mehta S., Loegering D.J., Mazurkiewicz J.E., Lennartz M.R. (2000) Differential requirement for classic and novel PKC isoforms in respiratory burst and phagocytosis in RAW 264.7 cells. Journal of Immunology. **165**: 2809.

268. Chanock S.J., El Benna J., Smith R.M., Babior B.M. (1994) The respiratory burst oxidase. Journal of Biological Chemistry. **269**: 24519.

269. DeLeo F.R., Quinn M.T. (1996) Assembly of the phagocyte NADPH oxidase: molecular interaction of oxidase proteins. Journal of Leukocyte Biology. **60**: 677.

270. Zu Y.L., Qi J., Gilchrist A., Fernandez G.A., Vazquez-Abad D., Kreutzer D.L., Huang C.K., Sha'afi R.I. (1998) p38 mitogen-activated protein kinase activation is required for human neutrophil function triggered by TNF-alpha or FMLP stimulation. Journal of Immunology. **160**: 1982.

271. Zu Y.L., Ai Y., Gilchrist A., Labadia M.E., Sha'afi R.I., Huang C.K. (1996) Activation of MAP kinase-activated protein kinase 2 in human neutrophils after phorbol ester or fMLP peptide stimulation. Blood. **87**: 5287.

272. Rose D.M., Winston B.W., Chan E.D., Riches D.W., Gerwins P., Johnson G.L., Henson P.M. (1997) Fc gamma receptor cross-linking activates p42, p38, and JNK/SAPK mitogen-activated protein kinases in murine macrophages: role for p42MAPK in Fc gamma receptor-stimulated TNF-alpha synthesis. Journal of Immunology. **158**: 3433.

273. Park H.S., Lee S.M., Lee J.H., Kim Y.S., Bae Y.S., Park J.W. (2001) Phosphorylation of the leucocyte NADPH oxidase subunit p47(phox) by casein kinase 2: conformation-dependent phosphorylation and modulation of oxidase activity. Biochemical Journal. **358**: 783.

274. Abo A., Webb M.R., Grogan A., Segal A.W. (1994) Activation of NADPH oxidase involves the dissociation of p21rac from its inhibitory GDP/GTP exchange protein (rhoGDI) followed by its translocation to the plasma membrane. Biochemical Journal. **298 Pt 3**: 585.

275. Gabig T.G., Crean C.D., Mantel P.L., Rosli R. (1995) Function of wild-type or mutant Rac2 and Rap1a GTPases in differentiated HL60 cell NADPH oxidase activation. Blood. **85**: 804.

276. Heyworth P.G., Knaus U.G., Settleman J., Curnutte J.T., Bokoch G.M. (1993) Regulation of NADPH oxidase activity by Rac GTPase activating protein(s). Molecular Biology of the Cell. **4**: 1217.

277. Knaus U.G., Heyworth P.G., Evans T., Curnutte J.T., Bokoch G.M. (1991) Regulation of phagocyte oxygen radical production by the GTP-binding protein Rac 2. Science. **254**: 1512.

278. Seifert R., Rosenthal W., Schultz G. (1986) Guanine nucleotides stimulate NADPH oxidase in membranes of human neutrophils. FEBS Letters. **205**: 161.

279. Seifert R., Rosenthal W., Schultz G., Wieland T., Gierschick P., Jakobs K.H. (1988) The role of nucleoside-diphosphate kinase reactions in G protein activation of NADPH oxidase by guanine and adenine nucleotides. European Journal of Biochemistry. **175**: 51.

280. Wolfl J., Dagher M.C., Fuchs A., Geiszt M., Ligeti E. (1996) In vitro activation of the NADPH oxidase by fluoride. Possible involvement of a factor activating GTP hydrolysis on Rac (Rac-GAP),. European Journal of Biochemistry. **239**: 369.

281. Kim C., Dinauer M.C. (2001) Rac2 is an essential regulator of neutrophil nicotinamide adenine dinucleotide phosphate oxidase activation in response to specific signaling pathways. Journal of Immunology. **166**: 1223.

282. Kwong C.H., Malech H.L., Rotrosen D., Leto T.L. (1993) Regulation of the human neutrophil NADPH oxidase by rho-related G- proteins. Biochmistry. **32**: 5711.

283. Williams D.A., Tao W., Yang F., Kim C., Gu Y., Mansfield P., Levine J.E., Petryniak B., Derrow C.W., Harris C., Jia B., Zheng Y., Ambruso D.R., Lowe J.B., Atkinson S.J., Dinauer M.C., Boxer L. (2000) Dominant negative mutation of the hematopoietic-specific Rho GTPase, Rac2, is associated with a human phagocyte immunodeficiency. Blood. **96**: 1646.

284. Dang P.M., Babior B.M., Smith R.M. (1999) NADPH dehydrogenase activity of p67PHOX, a cytosolic subunit of the leukocyte NADPH oxidase. Biochemistry. **38**: 5746.

285. Dang P.M., Johnson J.L., Babior B.M. (2000) Binding of nicotinamide adenine dinucleotide phosphate to the tetratricopeptide repeat domains at the N-terminus of p67PHOX, a subunit of the leukocyte nicotinamide adenine dinucleotide phosphate oxidase. Biochemistry. **39**: 3069.

286. Smith R.M., Curnutte J.T., Mayo L.A., Babior B.M. (1989) Use of an affinity label to probe the function of the NADPH binding component of the respiratory burst oxidase of human neutrophils. Journal of Biological Chemistry. **264**: 12243.

287. Smith R.M., Connor J.A., Chen L.M., Babior B.M. (1996) The cytosolic subunit p67phox contains an NADPH-binding site that participates in catalysis by the leukocyte NADPH oxidase. Journal of Clinical Investigation. **98**: 977.

288. Bromberg Y., Pick E. (1984) Unsaturated fatty acids stimulate NADPH-dependent superoxide production by cell-free system derived from macrophages. Cellular Immunolology. **88**: 213.

289. McPhail L.C., Shirley P.S., Clayton C.C., Snyderman R. (1985) Activation of the respiratory burst enzyme from human neutrophils in a cell-free system. Evidence for a soluble cofactor. Journal of Clinical Investigation. **75**: 1735.

290. Ischiropoulos H., Zhu L., Chen J., Tsai M., Martin J.C., Smith C.D., Beckman J.S. (1992) Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. Archives of Biochemistry and Biophysics. **298**: 431.

291. Ischiropoulos H., Zhu L., Beckman J.S. (1992) Peroxynitrite formation from macrophage-derived nitric oxide. Archives of Biochemistry and Biophysics. **298**: 431.

292. Lefkowitz D.L., Mills K., Morgan D., Lefkowitz S.S. (1992) Macrophage activation and immunomodulation by myeloperoxidase. Proceedings of the Society for Experimental Biology and Medicine. **199**: 204.

293. Lefkowitz D.L., Mills K.C., Moguilevsky N., Bollen A., Vaz A., Lefkowitz S.S. (1993) Regulation of macrophage function by human recombinant myeloperoxidase. Immunology Letters. **36**: 43.

294. Leung K.P., Goren M.B. (1989) Uptake and utilization of human polymorphonuclear leukocyte granule myeloperoxidase by mouse peritoneal macrophages. Cell and Tissue Research. **257**: 653.

295. Cassatella M.A., Della B.V., Berton G., Rossi F. (1985) Activation by gamma interferon of human macrophage capability to produce toxic oxygen molecules is accompanied by decreased Km of the superoxide-generating NADPH oxidase. Biochemical and Biophysical Research Communications. **132**: 908.

296. Sasada M., Pabst M.J., Johnston R.B., Jr. (1983) Activation of mouse peritoneal macrophages by lipopolysaccharide alters the kinetic parameters of the superoxide-producing NADPH oxidase. Journal of Biological Chemistry. **258**: 9631.
297. Tsunawaki S., Nathan C.F. (1984) Enzymatic basis of macrophage activation. Kinetic analysis of superoxide production in lysates of resident and activated mouse peritoneal macrophages and granulocytes. Journal of Biological Chemistry. **259**: 4305.
298. Cassatella M.A., Bazzoni F., Flynn R.M., Dusi S., Trinchieri G., Rossi F. (1990) Molecular basis of interferon-gamma and lipopolysaccharide enhancement of phagocyte respiratory burst capability. Studies on the gene expression of several NADPH oxidase components. Journal of Biological Chemistry. **265**: 20241.
299. Newburger P.E., Ezekowitz R.A., Whitney C., Wright J., Orkin S.H. (1988) Induction of phagocyte cytochrome b heavy chain gene expression by interferon gamma. Proceedings of the National Academy of Sciences of the United States of America. **85**: 5215.
300. Ding A.H., Nathan C.F., Stuehr D.J. (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. Journal of Immunology. **141**: 2407.
301. Green S.P., Hamilton J.A., Uhlinger D.J., Phillips W.A. (1994) Expression of p47-phox and p67-phox proteins in murine bone marrow- derived macrophages: enhancement by lipopolysaccharide and tumor necrosis factor alpha but not colony stimulating factor 1. Journal of Leukocyte Biology. **55**: 530.
302. Chaudhri G., Clark I.A. (1989) Reactive oxygen species facilitate the in vitro and in vivo lipopolysaccharide induced release of tumor necrosis factor. Journal of Immunology. **143**: 1290.
303. Ward R.A., Nakamura M., McLeish K.R. (2000) Priming of the neutrophil respiratory burst involves p38 mitogen- activated protein kinase-dependent exocytosis of flavocytochrome b558- containing granules. Journal of Biological Chemistry. **275**: 36713.
304. Kemmerich B., Rossing T.H., Pennington J.E. (1987) Comparative oxidative microbicidal activity of human blood monocytes and alveolar macrophages and activation by recombinant gamma interferon. The American Review of Respiratory Disease. **136**: 266.
305. Phillips W.A., Hamilton J.A. (1989) Phorbol ester-stimulated superoxide production by murine bone marrow- derived macrophages requires preexposure to cytokines. Journal of Immunology. **142**: 2445.
306. Sanchez-Mejorada G., Rosales C. (1988) Signal transduction by immunoglobulin Fc receptors. Journal of Leukocyte Biology. **63**: 521.
307. Zheleznyak A., Brown E.J. (1992) Immunoglobulin-mediated phagocytosis by human monocytes requires protein kinase C activation. Evidence for protein kinase C translocation to phagosomes. Journal of Biological Chemistry. **267**: 12042.

308. Allen L.A., Aderem A. (1996) Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. Journal of Experimental Medicine. **184**: 627.

309. Allen L.H., Aderem A. (1995) A role for MARCKS, the alpha isozyme of protein kinase C and myosin I in zymosan phagocytosis by macrophages. Journal of Experimental Medicine. **182**: 829.

310. Chan H.T., Kedzierska K., O'Mullane J., Crowe S.M., Jaworowski A. (2001) Quantifying complement-mediated phagocytosis by human monocyte-derived macrophages. Immunology and Cell Biology. **79**: 429.

311. Fallman M., Gullberg M., Hellberg C., Andersson T. (1992) Complement receptor-mediated phagocytosis is associated with accumulation of phosphatidylcholine-derived diglyceride in human neutrophils. Involvement of phospholipase D and direct evidence for a positive feedback signal of protein kinase. Journal of Biological Chemistry. **267**: 2656.

312. Secombes C.J., Cross A.R., Sharp G.J., Garcia R. (1992) NADPH oxidase-like activity in rainbow trout *Oncorhynchus mykiss* (Walbaum) macrophages. Developmental and Comparative Immunology. **16**: 405.

313. Itou T., Iida T., Kawatsu H. (1998) Evidence for the existence of cytochrome b558 in fish neutrophils by polyclonal anti-peptide antibody. Developmental and Comparative Immunology. **22**: 433.

314. Graham S., Secombes C. (1988) The production of a macrophage-activating factor from rainbow trout *Salmo gairdneri* leukocytes. Immunology. **65**: 293.

315. Graham S., Secombes C.J. (1990) Cellular requirements for lymphokine secretion by rainbow trout Salmo gairdneri leukocytes. Developmental and Comparative Immunology. **14**: 59.

316. Graham S., Secombes C.J. (1990) Do fish lymphocytes secrete interferon-gamma ? Journal of Fish Biology. **36**: 563.

317. Jang S.I., Hardie L.J., Secombes C.J. (1995) Elevation of rainbow trout *Oncorchynchus mykiss* macrophage respiratory burst activity with macrophage-derived supernatants. Journal of Leukocyte Biology. **57**: 943.

318. Neumann N.F., Fagan D., Belosevic M. (1995) Macrophage activating factor(s) secreted by mitogen stimulated goldfish kidney leukocytes synergize with bacterial lipopolysaccharide to induce nitric oxide production in teleost macrophages. Developmental and Comparative Imunology. **19**: 473.

319. Neumann N.F., Belosevic M. (1996) Deactivation of primed respiratory burst response of goldfish macrophages by leukocyte-derived macrophage activating factor(s). Developmental and Comparative Imunology. **20**: 427.

320. Novoa B., Figueras A., Ashton I., Secombes C.J. (1996) In vitro studies on the regulation of rainbow trout (*Oncorhynchus mykiss*) macrophage respiratory burst activity. Developmental and Comparative Immunology. **20**: 207.

321. Neumann N.F., Barreda D., Belosevic M. (1998) Production of a macrophage growth factor(s) by a goldfish macrophage cell line and macrophages derived from goldfish kidney leukocytes. Developmental and Comparative Imunology. **4**: 417.

322. Neumann N.F., Stafford J.L., Belosevic M. (2000) Biochemical and functional characterization of macrophage stimulating factors secreted by mitogen-induced goldfish kidney leukocytes. Fish and Shellfish Immunology. **10**: 167.

323. Stafford J.L., Neumann N.F., Belosevic M. (1999) Inhibition of macrophage activity by mitogen-induced goldfish leukocyte deactivating factor. Developmental and Comparative Imunology. **23**: 585.

324. Chen D.X., Ainsworth A.J. (1991) Assessment of metabolic activation of channel catfish peripheral blood neutrophils. Developmental and Comparative Immunology. **15**: 201.

325. Tafalla C., Novoa B. (2000) Requirements for nitric oxide production by turbot (*Scophthalmus maximus*) head kidney macrophages. Developmental and Comparative Immunology. **24**: 623.

326. Qin Q.W., Ototake M., Noguchi K., Soma G., Yokomizo Y., Nakanishi T. (2001) Tumor necrosis factor alpha (TNFalpha)-like factor produced by macrophages in rainbow trout, *Oncorhynchus mykiss*. Fish & Shellfish Immunology. **11**: 245.

327. Jang S.I., Hardie L.J., Secombes C.J. (1994) Effects of transforming growth factor-beta1 on rainbow trout *Oncorhynchis mykiss* macrophage respiratory burst activity. Developmental and Comparative Immunology. **4**: 315.

328. Tafalla C., Novoa B., Figueras A. (1999) Suppressive effect of turbot (*Scophthalmus maximus*) leukocyte-derived supernatants on macrophage and lymphocyte functions. Fish & Shellfish Immunology. **9**: 157.

329. Chung S., Secombes C.J. (1987) Activation of rainbow trout macrophages. Journal of Fish Biology. **31(Suppl. A)**: 51.

330. Nakagawara A., DeSantis N.M., Nogueira N., Nathan C.F. (1982) Lymphokines enhance the capacity of human monocytes to secret reactive oxygen intermediates. Journal of Clinical Investigation. **70**: 1042.

331. Francis C.H., Ellis A. (1994) Production of a lymphokine (macrophage activating factor) by salmon (*Salmo salar*) leukocytes stimulated with outer membrane protein antigens of *Aeromonas salmonicida*. Fish & Shellfish Immunology. **4**: 489.

332. Yin Z., Lam T.J., Sin Y.M. (1997) Cytokine-mediated antimicrobial immune response of catfish, *Clarias gariepinus*, as a defence against *Aeromonas hydrophila*. Fish & Shellfish Immunology. **7**: 93.

333. Secombes C.J., Laird L.M., Priede I.G. (1987) Lymphokine-release from rainbow trout leucocytes stimulated with concanavalin A. Effects upon macrophage spreading and adherence. Developmental and Comparative Immunology. **11**: 513.

334. Neumann N.F. (1999) Regulation of Antimicrobial Mechanisms of Macrophages of the Goldfish (*Carassius auratus*). Ph.D. Thesis Dissertation. University of Alberta, Edmonton, Alberta, Canada.

335. Barreda D., Neumann N.F., Belosevic M. (2000) Flow cytometric analysis of PKH26-labeled goldfish kidney-derived macrophages. Developmental and Comparative Imunology. **24**: 395.

336. Neumann N.F., Barreda D., Belosevic M. (2000) Generation and functional analysis of distinct macrophage sub-populations from goldfish (*Carassius Auratus* L.) kidney leukocyte cultures. Fish & Shellfish Immunology. **10**: 1.
337. Stafford J.L., McLauchlan P.E., Secombes C.J., Ellis A., Belosevic M. (2001) Generation of primary monocyte-like cultures from rainbow trout head kidney leukocytes. Developmental and Comparative Immunology. **25**: 447.
338. Marsden M.J., Secombes C.J. (1997) The influence of vaccine preparations on the induction of antigen specific responsiveness in rainbow trout *Oncorhynchus mykiss*. Fish & Shellfish Immunology. **7**: 455.
339. Marsden M.J., Cox D., Secombes C.J. (1994) Antigen-induced release of macrophage activating factor from rainbow trout *Oncorhynchus mykiss* leucocytes. Veterinary Immunology and Immunopathology. **42**: 199.
340. Mulero V., Meseguer J. (1998) Functional characterisation of a macrophage-activating factor produced by leucocytes of gilthead seabream (*Sparus aurata* L.). Fish & Shellfish Immunology. **8**: 143.
341. MacKenzie S., Planas J.V., Goetz F.W. (2003) LPS-stimulated expression of tumor necrosis factor-alpha mRNA in primary monocytes and in vitro differentiated macrophages. Developmental and Comparative Imunology. **27**: 393.
342. Nathan C.F., Murray H.W., Wiebe M.E., Rubin B.Y. (1983) Identification of interferon-gamma as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. Journal of Experimental Medicine. **158**: 670.
343. Cassatella M.A., Cappelli R., Della B.V., Grzeskowiak M., Dusi S., Berton G. (1988) Interferon-gamma activates human neutrophil oxygen metabolism and exocytosis. Immunology. **63**: 499.
344. Waterstrat P.R., Ainsworth A.J., Capley G. (1991) In vitro responses of channel catfish, *Ictalurus punctatus* , neutrophils to *Edwardsiella ictaluri*. Developmental and Comparative Immunology. **15**: 53.
345. Campos-Perez J.J., Ellis A.E., Secombes C.J. (1997) Investigation of factors influencing the ability of *Renibacterium salmoninarum* to stimulate rainbow trout macrophage respiratory burst activity. Fish & Shellfish Immunology. **7**: 555.
346. Solem S.T., Jorgensen J.B., Robertsen B. (1995) Stimulation of respiratory burst and phagocytic activity in Atlantic salmon (*Salmo salar* L.) macrophages by lipopolysaccharide. Fish & Shellfish Immunology. **5**: 475.
347. Taylor M.J., Hoole D. (1995) The chemiluminescence of cyprinid leucocytes in response to zymosan and extracts of *Ligula intestinalis* (Cestoda). Fish & Shellfish Immunology. **5**: 191.
348. Brattgjerd S., Evensen O., Lauve A. (1994) Effect of injected yeast glucan on the activity of macrophages in Atlantic salmon, *Salmo salar* L., as evaluated by in vitro hydrogen peroxide production and phagocytic capacity. Immunology. **83**: 288.
349. Dalmo R.A., Bogwald J., Ingebrigtsen K., Seljelid R. (1996) The immunomodulatory effect of laminaran (beta(1,3)-D-glucan) on Atlantic

salmon, *Salmo salar* L., anterior kidney leucocytes after intraperitoneal, peroral and peranal administration. Journal of Fish Diseases. **19**: 449.

350. Jorgensen J.B., Robertsen B. (1995) Yeast beta-glucan stimulates respiratory burst activity of Atlantic salmon (*Salmo salar* L.) macrophages. Developmental and Comparative Immunology. **19**: 43.

351. Robertsen B. (1999) Modulation of the non-specific defence of fish by structurally conserved microbial polymers. Fish & Shellfish Immunology. **9**: 269.

352. Tahir A., Secombes C.J. (1996) Modulation of dab (*Limanda limanda*, L.) macrophage respiratory burst activity. Fish & Shellfish Immunology. **6**: 135.

353. Enane N.A., Frenkel K., O' Connor J.M., Squibb K.S., Zelikoff J.T. (1993) Biological markers of macrophage activation: Applications for fish phagocytes. Immunology. **80**: 68.

354. Itou T., Iida T., Kawatsu H. (1996) Kinetics of oxygen metabolism during respiratory burst in Japanese eel neutrophils. Developmental and Comparative Immunology. **20**: 323.

355. Hardie L.J., Chappell L.H., Secombes C.J. (1994) Human tumor necrosis factor alpha influences rainbow trout *Oncorhynchus mykiss* leukocyte responses. Veterinary Immunology and Immunopathology. **40**: 73.

356. Jang S.I., Mulero V., Hardie L.J., Secombes C.J. (1995) Inhibition of rainbow trout phagocyte responsiveness to human tumor necrosis factor alpha (hTNF alpha) with monoclonal antibodies to the hTNF alpha 55 kDa receptor. Fish & Shellfish Immunology. **5**: 61.

357. Hirono I., Nam B., Kurobe Y., Aoki T. (2000) Molecular cloning, characterization and expression of tumor necrosis factor (TNF) cDNA and gene from Japanese flounder *Paralichthys alivaceus*. Journal of Immunology. **165**: 4423.

358. Laing K.J., Wang T., Zou J.J., Holland J., Hirono I., Aoki T., Secombes C.J. (2000) Cloning and expression analysis of *Oncorhynchus mykiss* tumor necrosis factor-alpha. European Journal of Biochemistry. **268**: 1315.

359. Zou J., Wang T., Hirono I., Aoki T., Inagawa H., Honda T., Soma G.I., Ototake M., Nakanishi T., Ellis A.E., Secombes C.J. (2002) Differential expression of two tumor necrosis factor genes in rainbow trout, *Oncorhynchus mykiss*. Developmental and Comparative Immunology. **26**: 161.

360. Park C.I., Kurobe T., Hirono I., Aoki T. (2003) Cloning and characterization of cDNAs for two distinct tumor necrosis factor receptor superfamily genes from Japanese flounder *Paralichthys olivaceus*. Developmental and Comparative Immunology. **27**: 365.

361. Saeij J.P., Stet R.J.M., de Vries B.J., van Muiswinkel W.B., Wiegertjes G.F. (2003) Molecular and functional characterization of carp TNF: a link between TNF polymorphism and trypanotolerance? Developmental and Comparative Immunology. **27**: 29.

362. Zou J., Secombes C.J., Long S., Miller N., Clem L.W., Chinchar V.G. (2003) Molecular identification and expression analysis of tumor necrosis factor in channel catfish (*Ictalurus punctatus*). Developmental and Comparative Immunology. **27**: 845.

363. Zou J., Peddie S., Scapigliati G., Zhang Y., Bols N.C., Ellis A.E., Secombes C.J. (2003) Functional characterisation of the recombinant tumor necrosis factors in rainbow trout, *Oncorhynchus mykiss*. Developmental and Comparative Immunology. **27**: 813

364. Bird S., Zou J., Wang T., Munday B., Cunningham C., Secombes C.J. (2002) Evolution of interleukin-1 beta. Cytokine and Growth Factor Reviews. **13**: 483.

365. Brubacher J.L., Secombes C.J., Zou J., Bols N.C. (2000) Constitutive and LPS-induced gene expression in a macrophage-like cell line from the rainbow trout (*Oncorhynchus mykiss*). Developmental and Comparative Immunology. **24**: 565.

366. Secombes C.J., Bird S., Cunningham C., Zou J. (1999) Interleukin-1 in fish. Fish & Shellfish Immunology. **9**: 335.

367. Zou J., Cunningham C., Secombes C.J. (1999) The rainbow trout *Oncorhynchus mykiss* interleukin-1beta gene has a different organization to mammals and undergoes incomplete splicing. European Journal of Biochemistry. **259**: 901.

368. Zou J., Wang T., Hong S., Cunningham C., Secombes C.J. (2000) Role of interleukin-1 beta in immune responses of rainbow trout. Fish & Shellfish Immunology. **10**: 289.

369. Zou J., Grabowski P.S., Cunningham C., Secombes C.J. (1999) Molecular cloning of interleukin 1beta from rainbow trout *Oncorhynchus mykiss* reveals no evidence of an ice cut site. Cytokine. **11**: 552.

370. Scapigliati G., Buonocore F., Bird S., Zou J., Pelegrin P., Falasca C., Prugnoli D., Secombes C.J. (2001) Phylogeny of cytokines: molecular cloning and expression analysis of sea bass *Dicentrarchus labrax* interleukin-1 beta. Fish & Shellfish Immunology. **11**: 711.

371. Pelegrin P., Garcia-Castillo J., Mulero V., Meseguer J. (2001) Interleukin-1 isolated from a marine fish reveals up regulated expression in macrophages following activation with lipopolysaccharide and lymphokines. Cytokine. **16**: 67.

372. Fujiki K., Shin D., Nakao M., Yano T. (2000) Molecular cloning and expression analysis of carp (*Cyprinus carpio*) interleukin-1 beta , high affinity immunoglobulin E Fc receptor gamma subunit and serum amyloid A. Fish & Shellfish Immunology. **10**: 229.

373. Engelsma M.Y., Stet R., Schipper H., Verburg-van Kemenade B. (2001) Regulation of interleukin-1 beta RNA expression in the common carp. Developmental and Comparative Immunology. **25**: 195.

374. Bird S., Wang T., Zou J., Cunningham C., Secombes C.J. (2000) The first cytokine sequence within cartilaginous fish: interleukin-1 in the small spotted catshark (*Scyliorhinus canicula*). Journal of Immunology. **168**: 3329.

375. Engelsma M.Y., Stet R.J.M., Saeij J.P.J., Verburg-van Kemenade B.M.L. (2003) Differential expression and haplotypic variation of two interleukin-1 genes in the common carp (*Cyprinus carpio* L.). Cytokine. **(in press)**.

376. Ferrante A. (1992) Activation of neutrophils by interleukins-1 and -2 and tumor necrosis factors. Immunology Series. **57**: 417.

377. Yagisawa M., Yuo A., Kitagawa S., Yazaki Y., Togawa A., Takaku F. (1995) Stimulation and priming of human neutrophils by IL-1 alpha and IL-1 beta: complete inhibition by IL-1 receptor antagonist and no interaction with other cytokines. Experimental Hematology. **23**: 603.

378. Sample A.K., Czuprynski C.J. (1994) Bovine neutrophil chemiluminescence is preferentially stimulated by homologous IL-1, but inhibited by the human IL-1 receptor antagonist. Veterinary Immunology and Immunopathology. **41**: 165.

379. Peddie S., Zou J., Cunningham C., Secombes C.J. (2001) Rainbow trout (*Oncorhynchus mykiss*) recombinant IL-1 beta and derived peptides induce migration of head kideny leukocytes in vitro. Fish & Shellfish Immunology. **11**: 697.

380. Hong S., Zou J., Crampe M., Peddie S., Scapigliati G., Secombes C.J. (2001) The production and bioactivity testing of rainbow trout (*Oncorhynchus mykiss)* recombinant IL-1beta. Veterinary Immunology and Immunopathology. **81**: 1.

381. Hong S., Peddie S., Campos-Pérez J.J., Zou J., Secombes C.J. (2003) The effect of intraperitoneally administered recombinant IL-1beta on immune parameters and resistance to *Aeromonas salmonicida* in the rainbow trout (*Oncorhynchus mykiss*). Developmental and Comparative Imunology. **27**: 801.

382. Calduch-Giner J.A., Sitja B.A., Alvarez P.P., Perez S.J. (1997) Growth hormone as an in vitro phagocyte-activating factor in the gilthead sea bream (*Sparus aurata*). Cell and Tissue Research. **287**: 535.

383. Munoz J., Esteban M.A., Meseguer J. (1999) In vitro culture requirements of sea bass (*Dicentrarchus labrax* L.) blood cells: Differential adhesion and phase contrast microscopic study. Fish & Shellfish Immunology. **9**: 417.

384. Munoz P., Calduch-Giner J.A., Sitja-Bobadilla A., Alvarez-Pellitero P., Perez-Sanchez J. (1998) Modulation of the respiratory burst activity of Mediterranean sea bass (*Dicentrarchus labrax* L.) phagocytes by growth hormone and parasitic status. Fish & Shellfish Immunology. **8**: 25.

385. Kitlen J.W., Hejbol E.K., Zinck T., Varming K., Byatt J.C., McLean E. (1997) Growth performance and respiratory burst activity in rainbow trout treated with growth hormone and vaccine. Fish & Shellfish Immunology. **7**: 297.

386. Hardie L.J., Ellis A.E., Secombes C.J. (1996) *In vitro* activation of rainbow trout macrophages stimulates inhibition of Renibacterium salmoninarum growth concomitant with augmented generation of respiratory burst products. Diseases of Aquatic Organisms. **25**: 175.

387. Green L.C., Ruiz D.L., Wagner D.A., Rand W., Istfan N., Young V.R., Tannenbaum S.R. (1981) Nitrate biosynthesis in man. Proceedings of the National Academy of Sciences of the United States of America. **78**: 7764.

388. Green L.C., Tannenbaum S.R., Goldman P. (1981) Nitrate synthesis in the germfree and conventional rat. Science. **212**: 56.

389. Wagner D.A., Young V.R., Tannenbaum S.R. (1983) Mammalian nitrate biosynthesis: incorporation of 15NH3 into nitrate is enhanced by endotoxin

treatment. Proceedings of the National Academy of Sciences of the United States of America. **80**: 4518.

390. Stuehr D.J., Marletta M.A. (1985) Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. Proceedings of the National Academy of Sciences of the United States of America. **82**: 7738.
391. James S.L. (1995) Role of nitric oxide in parasitic infections. Microbiology Reviews. **59**: 533.
392. Hibbs J.B.J. (1991) Synthesis of nitric oxide from L-arginine: a recently discovered pathway induced by cytokines with antitumour and antimicrobial activity. Research Immunology. **142**: 565.
393. Nathan C., Xie Q.W. (1991) Nitric oxide synthases: roles, Tolls, and controls. Cell. **78**: 915.
394. Nathan C.F. (1995) Natural resistance and nitric oxide. Cell. **82**: 873.
395. Nathan C.F., Hibbs J.B.J. (1991) Role of nitric oxide synthesis in macrophage antimicrobial activity. Current Opinion in Immunology. **3**: 65.
396. Stuehr D.J., Nathan C.F. (1989) Nitric oxide. A macrophage product responsible for cytostasis and respiratory inhibition in tumor target cells. Journal of Experimental Medicine. **169**: 1543.
397. Xie Q., Nathan C. (1994) The high-output nitric oxide pathway: role and regulation. Journal of Leukocyte Biology. **56**: 576.
398. Cho H.J., Xie Q.W., Calaycay J., Mumford R.A., Swiderek K.M., Lee T.D., Nathan C. (1992) Calmodulin is a subunit of nitric oxide synthase from macrophages. Journal of Experimental Medicine. **176**: 599.
399. Baek K.J., Thiel B.A., Lucas S., Stuehr D.J. (1993) Macrophage nitric oxide synthase subunits. Purification, characterization, and role of prosthetic groups and substrate in regulating their association into a dimeric enzyme. Journal of Biological Chemistry. **268**: 21120.
400. Stuehr D.J., Kwon N.S., Nathan C.F. (1990) FAD and GSH participate in macrophage synthesis of nitric oxide. Biochemical and Biophysical Research Communications. **168**: 558.
401. Stuehr D.J., Cho H.J., Kwon N.S., Weise M.F., Nathan C.F. (1991) Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. Proceedings of the National Academy of Sciences of the United States of America. **88**: 7773.
402. Stuehr D.J. (1997) Structure-function aspects in the nitric oxide synthases. Annual Review of Pharmacology and Toxicology. **37**: 339.
403. Tzeng E., Billiar T.R., Robbins P.D., Loftus M., Stuehr D.J. (1995) Expression of human inducible nitric oxide synthase in a tetrahydrobiopterin (H4B)-deficient cell line: H4B promotes assembly of enzyme subunits into an active dimer. Proceedings of the National Academy of Sciences of the United States of America. **92**: 11711.
404. Hibbs J.B.J., Vavrin Z., Taintor R.R. (1987) L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. Journal of Immunology. **138**: 550.

405. Iyengar R., Stuehr D.J., Marletta M.A. (1987) Macrophage synthesis of nitrite, nitrate, and N-nitrosamines: precursors and role of the respiratory burst. Proceedings of the National Academy of Sciences of the United States of America. **84**: 6369.

406. Nathan C.F. (1992) Nitric oxide as a secretory product of mammalian cells. FASEB Journal. **6**: 3051.

407. Shibazaki T., Fujiwara M., Sato H., Fujiwara K., Abe K., Bannai S. (1996) Relevance of the arginine transport activity to the nitric oxide synthesis in mouse peritoneal macrophages stimulated with bacterial lipopolysaccharide. Biochemistry and Biophysics Acta. **1311**: 150.

408. Xie L., Hattori Y., Tume N., Gross S.S. (2000) The preferred source of arginine for high-output nitric oxide synthesis in blood vessels. Seminars in Perinatology. **24**: 42.

409. Caivano M. (1998) Role of MAP kinase cascades in inducing arginine transporters and nitric oxide synthetase in RAW264 macrophages. FEBS Letters. **429**: 249.

410. Hammermann R., Dreissig M.D., Mossner J., Fuhrmann M., Berrino L., Gothert M., Racke K. (2000) Nuclear factor-kappa-beta mediates simultaneous induction of inducible nitric-oxide synthase and Up-regulation of the cationic amino acid transporter CAT-2-beta in rat alveolar macrophages. Molecular Pharmacology. **58**: 1294.

411. Alderton W.K., Cooper C.E., Knowles R.G. (2001) Nitric oxide synthases: structure, function and inhibition. Biochemical Journal. **357**: 593.

412. Holm P., Kankaanranta H., Oja S.S., Knowles R.G., Moilanen E. (1999) No detectable NO synthesis from L-arginine or N(G)-hydroxy-L-arginine in fMLP-stimulated human blood neutrophils despite production of nitrite, nitrate, and citrulline from N(G)-hydroxy-L-arginine. Journal of Leukocyte Biology. **66**: 127.

413. Schmidt H.H., Hofmann H., Schindler U., Shutenko Z.S., Cunningham D.D., Feelisch M. (1996) No .NO from NO synthase. Proceedings of the National Academy of Sciences of the United States of America. **93**: 14492.

414. Denicola A., Rubbo H., Rodriguez D., Radi R. (1993) Peroxynitrite-mediated cytotoxicity to Trypanosoma cruzi. Archives of Biochemistry and Biophysics. **304**: 279.

415. Zhu L., Gunn C., Beckman J.S. (1992) Bactericidal activity of peroxynitrite. Archives of Biochemistry and Biophysics. **298**: 452.

416. Xia Y., Roman L.J., Masters B.S., Zweier J.L. (1998) Inducible nitric-oxide synthase generates superoxide from the reductase domain. Journal of Biological Chemistry. **273**: 22635.

417. Xia Y., Zweier J.L. (1997) Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. Proceedings of the National Academy of Sciences of the United States of America. **94**: 6954.

418. Drapier J.C., Hibbs J.B.J. (1986) Murine cytotoxic activated macrophages inhibit aconitase in tumor cells. Inhibition involves the iron-sulfur prosthetic group and is reversible. Journal of Clinical Investigation. **78**: 790.

419. Drapier J.C., Pellat C., Henry Y. (1991) Generation of EPR-detectable nitrosyl-iron complexes in tumor target cells cocultured with activated macrophages. Journal of Biological Chemistry. **266**: 10162.

420. Drapier J.C., Hibbs J.B.J. (1996) Aconitases: a class of metalloproteins highly sensitive to nitric oxide synthesis. Methods in Enzymology. **269**: 26.

421. Gopalakrishna R., Chen Z.H., Gundimeda U. (1993) Nitric oxide and nitric oxide-generating agents induce a reversible inactivation of protein kinase C activity and phorbol ester binding. Journal of Biological Chemistry. **268**: 27180.

422. Griscavage J.M., Rogers N.E., Sherman M.P., Ignarro L.J. (1993) Inducible nitric oxide synthase from a rat alveolar macrophage cell line is inhibited by nitric oxide. Journal of Immunology. **151**: 6329.

423. Kwon N.S., Stuehr D.J., Nathan C.F. (1991) Inhibition of tumor cell ribonucleotide reductase by macrophage-derived nitric oxide. Journal of Experimental Medicine. **174**: 761.

424. Thomas S.R., Mohr D., Stocker R. (1994) Nitric oxide inhibits indoleamine 2,3-dioxygenase activity in interferon-gamma primed mononuclear phagocytes. Journal of Biological Chemistry. **269**: 14457.

425. Lee C., Miura K., Liu X., Zweier J.L. (2000) Biphasic regulation of leukocyte superoxide generation by nitric oxide and peroxynitrite. Journal of Biological Chemistry. **275**: 38965.

426. Assreuy J., Cunha F.Q., Liew F.Y., Moncada S. (1993) Feedback inhibition of nitric oxide synthase activity by nitric oxide. British Journal of Pharmacology. **108**: 833.

427. Griscavage J.M., Fukuto J.M., Komori Y., Ignarro L.J. (1994) Nitric oxide inhibits neuronal nitric oxide synthase by interacting with the heme prosthetic group. Role of tetrahydrobiopterin in modulating the inhibitory action of nitric oxide. Journal of Biological Chemistry. **269**: 21644.

428. Matthews J.R., Botting C.H., Panico M., Morris H.R., Hay R.T. (1996) Inhibition of NF-kappa-beta DNA binding by nitric oxide. Nucleic Acids Research. **24**: 2236.

429. Xie Q.W., Whisnant R., Nathan C. (1993) Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide. Journal of Experimental Medicine. **177**: 1779.

430. Xie Q.W., Kashiwabara Y., Nathan C. (1994) Role of transcription factor NF-kappa-beta/Rel in induction of nitric oxide synthase. Journal of Biological Chemistry. **269**: 4705.

431. Xie Q. (1997) A novel lipopolysaccharide-response element contributes to induction of nitric oxide synthase. Journal of Biological Chemistry. **272**: 14867.

432. Ganster R.W., Taylor B.S., Shao L., Geller D.A. (2001) Complex regulation of human inducible nitric oxide synthase gene transcription by Stat 1 and NF-kappa-beta. Proceedings of the National Academy of Sciences of the United States of America. **98**: 8638.

433. Ohmori Y., Schreiber R.D., Hamilton T.A. (1997) Synergy between interferon-gamma and tumor necrosis factor-alpha in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor kappa-beta. Journal of Biological Chemistry. **272**: 14899.

434. Yoza B.K., Hu J.Y., McCall C.E. (1996) Protein-tyrosine kinase activation is required for lipopolysaccharide induction of interleukin 1beta and NF kappa-beta activation, but not NF kappa-beta nuclear translocation. Journal of Biological Chemistry. **271**: 18306.

435. Kamijo R., Harada H., Matsuyama T., Bosland M., Gerecitano J., Shapiro D., Le J., Koh S.I., Kimura T., Green S.J., et. al. (1994) Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. Science. **263**: 1612.

436. Martin E., Nathan C., Xie Q.W. (1994) Role of interferon regulatory factor 1 in induction of nitric oxide synthase. Journal of Experimental Medicine. **180**: 977.

437. Salkowski C.A., Barber S.A., Detore G.R., Vogel S.N. (1996) Differential dysregulation of nitric oxide production in macrophages with targeted disruptions in IFN regulatory factor-1 and -2 genes. Journal of Immunology. **156**: 3107.

438. Kristof A.S., Marks-Konczalik J., Moss J. (2001) Mitogen-activated protein kinases mediate activator protein-1-dependent human inducible nitric-oxide synthase promoter activation. Journal of Biological Chemistry. **276**: 8445.

439. Feng G.J., Goodridge H.S., Harnett M.M., Wei X.Q., Nikolaev A.V., Higson A.P., Liew F.Y. (1999) Extracellular signal-related kinase (ERK) and p38 mitogen-activated protein (MAP) kinases differentially regulate the lipopolysaccharide- mediated induction of inducible nitric oxide synthase and IL-12 in macrophages: *Leishmania* phosphoglycans subvert macrophage IL-12 production by targeting ERK MAP kinase. Journal of Immunology. **163**: 6403.

440. Ropert C., Almeida I.C., Closel M., Travassos L.R., Ferguson M.A., Cohen P., Gazzinelli R.T. (2001) Requirement of mitogen-activated protein kinases and I kappa B phosphorylation for induction of proinflammatory cytokines synthesis by macrophages indicates functional similarity of receptors triggered by glycosylphosphatidylinositol anchors from parasitic protozoa and bacterial lipopolysaccharide. Journal of Immunology. **166**: 3423.

441. Amin A.R., Attur M., Vyas P., Leszczynska-Piziak J., Levartovsky D., Rediske J., Clancy R.M., Vora K.A., Abramson S.B. (1995) Expression of nitric oxide synthase in human peripheral blood mononuclear cells and neutrophils. Journal of Inflammation. **47**: 190.

442. Evans T.J., Buttery L.D., Carpenter A., Springall D.R., Polak J.M., Cohen J. (1996) Cytokine-treated human neutrophils contain inducible nitric oxide synthase that produces nitration of ingested bacteria. Proceedings of the National Academy of Sciences of the United States of America. **93**: 9553.

443. Webb J.L., Polak J.M., Evans T.J. (2001) Effect of adhesion on inducible nitric oxide synthase (iNOS) production in purified human neutrophils. Clinical & Experimental Immunology. **123**: 42.

444. Wheeler M.A., Smith S.D., Garcia-Cardena G., Nathan C.F., Weiss R.M., Sessa W.C. (1997) Bacterial infection induces nitric oxide synthase in human neutrophils. Journal of Clinical Investigation. **99**: 110.

445. MacMicking J., Xie Q.W., Nathan C. (1997) Nitric oxide and macrophage function. Annual Review in Immunology. **15**: 323.

446. Denis M. (1994) Human monocytes/macrophages: NO or no NO? Journal of Leukocyte Biology. **55**: 682.

447. Murray H.W., Teitelbaum R.F. (1992) L-arginine-dependent reactive nitrogen intermediates and the antimicrobial effect of activated human mononuclear phagocytes. Journal of Infectious Diseases. **165**: 513.

448. Schneemann M., Schoedon G., Hofer S., Blau N., Guerrero L., Schaffner A. (1993) Nitric oxide synthase is not a constituent of the antimicrobial armature of human mononuclear phagocytes [see comments]. Journal of Infectious Diseases. **167**: 1358.

449. Schneemann M., Schoedon G., Linscheid P., Walter R., Blau N., Schaffner A. (1997) Nitrite generation in interleukin-4-treated human macrophage cultures does not involve the nitric oxide synthase pathway. Journal of Infectious Diseases. **175**: 130.

450. Schoedon G., Schneemann M., Walter R., Blau N., Hofer S., Schaffner A. (1995) Nitric oxide and infection: another view. Clinical Infectious Diseases. **21 Suppl 2**: S152.

451. Bose M., Farnia P. (1995) Proinflammatory cytokines can significantly induce human mononuclear phagocytes to produce nitric oxide by a cell maturation-dependent process. Immunology Letters. **48**: 59.

452. Denis M. (1991) Tumor necrosis factor and granulocyte macrophage-colony stimulating factor stimulate human macrophages to restrict growth of virulent *Mycobacterium avium* and to kill avirulent *M. avium*: killing effector mechanism depends on the generation of reactive nitrogen intermediates. Journal of Leukocyte Biology. **49**: 380.

453. Munoz-Fernandez M.A., Fernandez M.A., Fresno M. (1992) Activation of human macrophages for the killing of intracellular *Trypanosoma cruzi* by TNF-alpha and IFN-gamma through a nitric oxide- dependent mechanism. Immunology Letters. **33**: 35.

454. Nicholson S., Bonecini-Almeida M.D., R. L.e.S.J., Nathan C., Xie Q.W., Mumford R., Weidner J.R., Calaycay J., Geng J., Boechat N. (1996) Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. Journal of Experimental Medicine. **183**: 2293.

455. Charles I.G., Palmer R.M., Hickery M.S., Bayliss M.T., Chubb A.P., Hall V.S., Moss D.W., Moncada S. (1993) Cloning, characterization, and expression of a cDNA encoding an inducible nitric oxide synthase from the human chondrocyte. Proceedings of the National Academy of Sciences of the United States of America. **90**: 11419.

456. Chartrain N.A., Geller D.A., Koty P.P., Sitrin N.F., Nussler A.K., Hoffman E.P., Billiar T.R., Hutchinson N.I., Mudgett J.S. (1994) Molecular cloning, structure, and chromosomal localization of the human inducible nitric oxide synthase gene. Journal of Biological Chemistry. **269**: 6765.

457. Geller D.A., Lowenstein C.J., Shapiro R.A., Nussler A.K., Di Silvio M., Wang S.C., Nakayama D.K., Simmons R.L., Snyder S.H., Billiar T.R. (1993) Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. Proceedings of the National Academy of Sciences of the United States of America. **90**: 3491.

458. Martin J.H., Edwards S.W. (1993) Changes in mechanisms of monocyte/macrophage-mediated cytotoxicity during culture. Reactive oxygen intermediates are involved in monocyte- mediated cytotoxicity, whereas reactive nitrogen intermediates are employed by macrophages in tumor cell killing. Journal of Immunology. **150**: 3478.

459. Martin J.H., Edwards S.W. (1994) Interferon-gamma enhances monocyte cytotoxicity via enhanced reactive oxygen intermediate production. Absence of an effect on macrophage cytotoxicity is due to failure to enhance reactive nitrogen intermediate production. Immunology. **81**: 592.

460. Hamerman J.A., Aderem A. (2001) Functional transitions in macrophages during *in vivo* infection with *Mycobacterium bovis* Bacillus Calmette-Guerin. Journal of Immunology. **167**: 2227.

461. Holmqvist B., Ekstrom P. (1997) Subcellular localization of neuronal nitric oxide synthase in the brain of a teleost: An immunoelectron and confocal microscopical study. Brain Research. **745**: 67-82.

462. Bruning G., Hattwig K., Mayer B. (1996) Nitric oxide synthase in the peripheral nervous system of the goldfish, *Carassius auratus*. Cell and Tissue Research. **284**: 87-98.

463. Schoor W.P., Plum J.A. (1994) Induction of nitric oxide synthase in channel catfish (*Ictalurus punctatus*) by *Edwardsiella ictaluri*. Diseases of Aquatic Organisms. **19**: 153.

464. Wang R., Neumann N.F., Shen Q., Belosevic M. (1995) Establishment and characterization of macrophage cell line from the goldfish. Fish & Shellfish Immunology. **5**: 329.

465. Laing K.J., Grabowski P.S., Belosevic M., Secombes C.J. (1996) A partial sequence for nitric oxide synthase from a goldfish (*Carassius auratus*) macrophage cell line. Immunology and Cell Biology. **74**: 374.

466. Laing K.J., Hardy L.J., Aartsen W., Grabowski P.S., Secombes C.J. (1999) Expression of an inducible nitric oxide synthase gene in rainbow trout *Oncorhynchus mykiss*. Developmental and Comparative Imunology. **23**: 71.

467. Campos-Perez J.J., Ward M., Grabowski R.S., Ellis A.E., Secombes C.J. (2000) The gills are an important site of iNOS expression in rainbow trout (*Oncorhynchu mykiss*) after challenge with Gram-negative pathogen *Renibacterium salmonarium*. Immunology. **99**: 153.

468. Barroso J.B., Carreras A., Esteban F.J., Peinado M.A., Martinez-Lara E., Valderrama R., Jimenez A., Rodrigo J., Lupianez J.A. (2000) Molecular and kinetic characterization and cell type location of inducible nitric oxide

synthase in fish. American Journal of Physiology. Regulatory Integrative and Comparative Physiology. **279**: R650-R6.

469. Wang T., Ward M., Grabowski P.S., Secombes C.J. (2001) Molecular cloning, gene organization and expression of rainbow trout (*Oncorhynchus mykiss*) inducible nitric oxide synthase (iNOS) gene. Biochemical Journal. **358**: 747.
470. Saeij J.P., Stet R.J., Groeneveld A., Verburg-van Kemenade L.B., van Muiswinkel W.B., Wiegertjes G.F. (2000) Molecular and functional chracterization of a fish inducible-type nitric oxide synthase. Immunogenetics. **51**: 339.
471. Shin D.H., Lim H.S., Cho S.K., Lee H.Y., Lee H.W., Lee K.H., Chung Y.H., Cho S.S., Ik Cha C., Hwang D.H. (2000) Immunocytochemical localization of neuronal and inducible nitric oxide synthase in the retina of zebrafish, *Brachydanio rerio*. Neuroscience Letters. **292**: 220.
472. Oyan A.M., Nilsen F., Goksoyr A., Holmqvist B. (2000) Partial cloning of constitutive and inducible nitric oxide synthases and detailed neuronal expression of NOS mRNA in the cerebellum and optic tectum of adult Atlantic salmon (*Salmo salar*). Brain Research. Molecular Brain Research. **78**: 38.
473. Green S.J., Crawford R.M., Hockmeyer J.T., Meltzer M.S., Nacy C.A. (1990) *Leishmania major* amastigotes initiate the L-arginine-dependent killing mechanism in IFN-gamma-stimulated macrophages by induction of tumor necrosis factor-alpha. Journal of Immunology. **145**: 4290.
474. Green S.J., Nacy C.A., Meltzer M.S. (1991) Cytokine-induced synthesis of nitrogen oxides in macrophages: a protective host response to *Leishmania* and other intracellular pathogens. Journal of Leukocyte Biology. **50**: 93.
475. Green S.J., Scheller L.F., Marletta M.A., Seguin M.C., Klotz F.W., Slayter M., Nelson B.J., Nacy C.A. (1994) Nitric oxide: cytokine-regulation of nitric oxide in host resistance to intracellular pathogens. Immunol Lett. **43**: 87.
476. Guy R.A., Belosevic M. (1995) Response of scid mice to establishment of *Leishmania major* infection. Clinical & Experimental Immunology. **100**: 440.
477. Stafford J.L., Belosevic M. (2003) Transferrin and the innate immune response of fish: identification of a novel mechanism of macrophage activation. Developmental and Comparative Immunology. **27**: 539.
478. Stafford J.L., Neumann N.F., Belosevic M. (2001) Products of proteolytic cleavage of transferrin induce nitric oxide response of goldfish macrophages. Developmental and Comparative Immunology. **25**: 101.
479. Klausner R.D., Ashwell G., Harford J.B., Bridges K.R. (1983) Binding of apotransferrin to K562 cells: explanation of the transferrin cycle. Proceedings of the National Academy of Sciences of the United States of America. **80**: 2263.
480. Klausner R.D., van Renswoude J., Ashwell G., Kempf C., Schechter A.N., Dean A., Bridges K.R. (1983) Receptor-mediated endocytosis of transferrin in K562 cells. Journal of Biological Chemistry. **258**: 4715.
481. Dautry-Varsat A., Ciechanover A., Lodish H.F. (1983) pH and the recycling of transferrin during receptor-mediated endocytosis. Proceedings of the National Academy of Sciences of the United States of America. **80**: 2258.

482. Ciechanover A., Schwartz A.L., Duatry-Varsat A., Lodish H.F. (1983) Kinetics of internalization and recycling of transferrin and the transferrin receptor in a human hepatoma cell line. Journal of Biological Chemistry. **258**: 968.
483. Hopkins C.R., Trowbridge I.S. (1983) Internalization and processing of transferrin and the transferrin receptor in human carcinoma A431 cells. Journal of Cellular Biology. **97**: 508.
484. Worwood M. (1989) An overview of iron metabolism at a molecular level. Journal of Internal Medicine. **226**: 381.
485. Baldwin G.S. (1993) Comparison of transferrin sequences from different species. Comparative Biochemistry and Physiology B. **106**: 203.
486. Anderson B.F., Baker H.M., Noris G.F., Rumball S.V., Baker E.N. (1990) Apolactoferrin structure demonstrates ligand-induced conformational change in transferrins. Nature. **344**: 784.
487. Retzer M.D., Kabani A., Button L.L., Yu R.H., Schryvers A.B. (1996) Production and characterization of chimeric transferrins for the determination of the binding domains for bacterial transferrin receptors. Journal of Biological Chemistry. **271**: 1166.
488. Martin A.W., Huebers E., Huebers H., Webb J., Finch C.A. (1984) A mon-sited transferin from a representative deuterostome: the ascidian *Pyura stolonifera* (subphylum *Urochordata*). Blood. **64**: 1047.
489. Jamroz R.C., Gasdaska J.R., Bradfield J.Y., Law J.H. (1993) Transferrin in a cockroach: molecular cloning, characterization, and suppression by juvenile hormone. Biochemistry. **90**: 1320.
490. Bartfeld N.S., Law J.H. (1990) Isolation and molecular cloning of transferrin from the tobacco hornworm, *Manduca sexta*. Sequence similarity to the vertebrate transferrins. Journal of Biological Chemistry. **15**: 21684.
491. Yoshiga T., Hernandez V.P., Fallon A.M., Law J.H. (1997) Mosquito transferrin, an acute-phase protein that is up-regulated upon infection. Proceedings of the National Academy of Sciences of the United States of America. **94**: 12337.
492. Ford M.J. (2001) Molecular evolution of transferrin: evidence for positive selection in salmonids. Molecular Biology and Evolution. **18**: 639.
493. Evans W.H., Wilson S.M., Bednarek J.M., Peterson E.A., Knight R.D., Mage M.G., McHugh L. (1989) Evidence for a factor in normal human serum that induces human neutrophilic granulocyte end-stage maturation *in vitro*. Leukemia Research. **13**: 673.
494. Breitman T.R., Collins S.J., Keene B.R. (1980) Replacement of serum by insulin and transferrin supports growth and differentiation of the human promyelocytic cell line, HL-60. Experimental Cell Research. **126**: 494.
495. Rossi M.C., Zetter B.R. (1992) Selective stimulation of prostatic carcinoma cell proliferation by transferrin. Proceedings of the National Academy of Sciences of the United States of America. **89**: 6197.
496. Wang L.G., Liu X.M., Wikiel H., Bloch A. (1995) Activation of casein kinase ll in ML-1 human myeloblastic leukemia cells requires IGF-1 and transferrin. Journal of Leukocyte Biology. **57**: 332.

497. Tang S., Leung J.C., Tsang A.W., Lan H.Y., Chan T.M., Lai K.N. (2002) Transferrin up-regulates chemokine synthesis by human proximal tubular epithelial cells: implication on mechanism of tubuloglomerular communication in glomerulopathic proteinura. Kidney International. **61**: 1655.

498. Takenaka K.V., Sakai N., Murase S., Kuroda T., Okumura A., Sawada M. (2000) Elevated transferrin concentration in cerebral spinal fluid after subarachnoid hemorrhage. Neurological Research. **22**: 797.

499. Weinzimer S.A., Gibson T.B., Collett-Solberg P.F., Khare A., Liu B., Cohen P. (2001) Transferrin is an insulin-like growth factor-binding protein-3 binding protein. Clinics in Endocrinology and Metabolism. **86**: 1806.

500. Sakamoto H., Sakamoto N., Oryu M., Kobayashi T., Ogawa Y., Ueno M., Shinnou M. (1997) A novel function of transferrin as a constituent of macromolecular activators of phagocytosis from platelets and their precursors. Biochemical and Biophysical Research Communications. **230**: 270.

501. Xie H., Rath N.C., Huff G.R., Balog J.M., Huff W.E. (2001) Inflammation-induced changes in serum modulate chicken macrophage function. Veterinary Immunology and Immunopathology. **80**: 225.

502. Xie H., Huff G.R., Huff W.E., Balog J.M., Rath N.C. (2002) Effects of ovotransferrin on chicken macrophages and heterophil-granulocytes. Developmental and Comparative Immunology. **26**: 805.

503. Ibrahim M.A., Iwamori E., Sugimoto Y., Aoki T. (1998) Identification of a distinct antibacterial domain within the N-lobe of ovatransferrin. Biochemical and Biophysical Acta. **1401**: 289.

504. Bayne C.J., Gerwick L., Fujiki K., Nakao M., Yano T. (2001) Immune-relevant (including acute phase) genes identified in the livers of rainbow trout, Oncorhynchus mykiss, by means of suppression subtractive hybridization. Developmental and Comparative Immunology. **25**: 205.

505. Basran G.S., Byrne A.J., Hardy J.G. (1985) A noninvasive technique for monitoring lung vascular permeability in man. Nuclear Medicine Communications. **6**: 3.

506. Bergmann H., Kolarz G. (1976) Pertechnetate uptake of joints in rheumatoid arthritis. European Journal of Nuclear Medicine. **1**: 205-10.

507. Colditz I.G., Altmann K.G., Watson D.L. (1992) Intradermal and percutaneous transudation of IgG1 and transferrin in sheep. Immunology and Cell Biology. **70 (Pt 5)**: 323.

508. Raijmakers P.G., Groeneveld A.B., Rauwerda J.A., Teule G.J., Hack C.E. (1997) Acute lung injury after aortic surgery: the relation between lung and leg microvascular permeability to 111indium-labelled transferrin and circulating mediators. Thorax. **52**: 866.

509. Chadzinska M., Scislowska-Czarnecka A., Plytycz B. (2000) Inhibitory effects of morphine on some inflammation-related parameters in the goldfish *Carassius auratus* L. Fish & Shellfish Immunology. **10**: 531.

510. Andreesen R., Osterholz J., Bodemann H., Bross K.J., Costabl U., Lohr G.W. (1984) Expression of transferrin receptors and intracellular ferritin during terminal differentiation of human monocytes. Blut. **49**: 195.

511. Andreesen R., Bross K.J., Osterholz J., Emmrich F. (1986) Human macrophage maturation and heterogeneity: analysis with a newly generated set of monoclonal antibodies to differentiation antigens. Blood. **67**: 1257.

512. Andreesen R., Boyce N.W., Atkins R.C. (1987) The expression of specific differentiation antigens on macrophages infiltrating rejecting renal allografts. Transplantation Proceedings. **19**: 2885.

513. Andreesen R., Sephton R.G., Gadd S., Atkins R.C., De Abrew S. (1988) Human macrophage maturation in vitro: expression of functional transferrin binding sites of high affinity. Blut. **57**: 77.

514. Beck G., Ellis T., Zhang H., Lin W., Beauregard K., Habicht G.S., Truong N. (2001) Nitric oxide production by coelomocytes of *asterias forbesi*. Developmental and Comparative Immunology. **25**: 1.

515. Torreilles J., Guerin M. (1999) Production of peroxynitrite by zymosan stimulation of *Mytilus galloprovincialis* haemocytes in vitro. Fish & Shellfish Immunology. **9**: 509.

516. Baker E.N., Lindley P.F. (1992) New perspectives on the structure and function of transferrins. Journal of Inorganic Biochemistry. **47**: 147.

517. Dall 'Asta V., Gatti R., Orlandini G., Rossi P., Rotoli B., Sala R., Bussolati O., Gazzola G. (1997) Membrane potential changes visualized in complete growth media through confocal laser scanning microscopy of bis-oxonol-loaded cells. Experimental Cell Research. **231**: 260.

518. Werner P.A.M., Galbraith R.M., Arnaud P. (1983) DEAE-Affi-Gel Blue chromatography of human serum: use for purification of native transferrin. Archives of Biochemistry and Biophysics. **1**: 393.

519. Yu R., Schryvers A.B. (1994) Transferrin receptors on ruminant pathogens vary in their interaction with the C-lobe and N-lobe of ruminant transferrins. Canadian Journal of Microbiology. **40**: 532.

520. Guy R.A., Belosevic M. (1993) Comparison of receptors required for the entry of *Leishmania major* amastigotes into macrophages. Infection and Immunity. **61**: 1553.

521. Bienek D.R., Plouffe D.A., Wiegertjes G.F., Belosevic M. (2002) Immunization of goldfish with excretory/secretory molecules of *Trypanosoma danilewskyi* confers protection against infection. Developmental and Comparative Immunology. **26**: 649.

522. Thompson J.D., Higgins D.G., Gibson T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignement through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research. **22**: 4673.

523. Schmidt H.A., Strimmer K., Vingron M., von Haeseler A. (2002) TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. Bioinformatics. **18**: 502.

524. Hirokawa T., Boon-Chieng S., Mitaku S. (1998) SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics. **14**: 378.

525. Sambrook J., Fritsch E.F., Maniatis T. (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory.

526. Schoner R.G., Ellis L.F., Schoner B.E. (1992) Isolation and purification of protein granules from *Escherichia coli* cells overproducing bovine growth hormone. Biotechnology. **24**: 349.

527. Hoefkens P., de Smit M.H., de Jeu-Jaspars N.M., Huijskes-Heins M.I., de Jong G., van Eijk H.G. (1996) Isolation, renaturation and partial characterization of recombinant human transferrin and its half molecules from *Escherichia coli*. The International Journal of Biochemistry & Cell Biology. **28**: 975.

528. Green L.C., Wagner D.A., Glogowski J., Skipper P.L., Wishnok J.S., Tannenbaum S.R. (1982) Analysis of nitrate, nitrite, and 15-nitrate in biological fluids. Analytical Biochemistry. **126**: 131.

529. Emmendorffer A., Hecht M., Lohmann-Matthes M.L., Roesler J. (1990) A fast and easy method to determine the production of reactive oxygen intermediates by human and murine phagocytes using dihydrorhodamine 123. Journal of Immunological Methods. **131**: 269.

530. Rothe G., Emmendorffer A., Oser A., Roesler J., Valet G. (1994) Flow cytometric measurement of the respiratory burst activity of phagocytes using dihydrorhodamine 123. Journal of Immunological Methods. **138**: 133.

531. Richardson M.O., Ayliffe M.J., Helbert M., Davies E.G. (1998) A simple flow cytometric assay using dihydrorhodamine for the measurement of the neutrophil respiratory burst in whole blood: comparison with the quantitative nitrobluetetrazolium test. Journal of Immunological Methods. **219**: 187.

532. van Furth R., Cohn Z.A., Hirsh J.G., Humphry J.H., Spector W.G., Langevoort H.L. (1972) The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursors. Bulletin WHO. **46**: 845.

533. Naito M., Umeda S., Yamamoto T., Moriyama H., Umezu H., Hasegawa G., Usuda H., Shultz L.D., Takahashi K. (1996) Development, differentiation, and phenotypic heterogeneity of murine tissue macrophages. Journal of Leukocyte Biology. **59**: 133.

534. Yamishita A. (1996) Role of yolk sac endodermal cells with special reference to the fetal macrophage differentiation. Journal of Leukocyte Biology. **59**: 139.

535. Alliot F., Lecain E., Grima B., Pessac B. (1991) Microglial progenitors with a high proliferative potential in the embryonic and adult mouse brain. Proceedings of the National Academy of Sciences of the United States of America. **88**: 1541.

536. Sawyer R.T., Strausbauch P.H., Volkman A. (1982) Resident macrophage proliferation in mice depleted of blood monocytes by strontium-89. Laboratroy Investigation. **46**: 165.

537. Shibata Y., Dempsey W.L., Morahan P.S., Volkman A. (1985) Selectively eliminated blood monocytes and splenic suppressor macrophages in mice depleted of bone marrow by strontium 89. Journal of Leukocyte Biology. **38**: 659.

538. Yamamada M., Naito M., Takahashi K. (1990) Kupffer cell proliferation and glucan-induced granuloma formation in mice depleted of blood monocytes by strontium 89. Journal of Leukocyte Biology. **47**: 195.

539. Virelizier J.L., Arenzana-Seisdedos F. (1985) Immunological functions of macrophages and their regulation by interferons. Medical Biology. **63**: 149.

540. Loms Ziegler-heitbrock H.W. (1989) The biology of the monocyte system. European Journal of Cell Biology. **49**: 1.

541. Stewart C.C., C. R.M., J. S.S. (1994) The proliferation and differentiation of macrophages. Immunology Series. **60**: 3.

542. Rutherford M.S., Witsell A., Schook L.B. (1993) Mechanisms generating functionally heterogeneous macrophages: chaos revisited. Journal of Leukocyte Biology. **53**: 602-18.

543. Donahue R.E., Seehra J., Metzger M., Lefebvre D., Rock B., Carbone S., Nathan D.G., Garnick M., Sehgal P.K., Laston D., LaVallie E., McCoy J., Schendel P.F., Norton C., Turner K., Yang Y.-C., Clark S.C. (1988) Human IL-3 and GM-CSF act synergistically in stimulating hematopoiesis in primates. Science. **241**: 1820.

544. Falk L.A., Vogel S. (1988) Granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (CSF-1) synergize to stimulate progenitor cells with high proliferative potential. Journal of Leukocyte Biology. **44**: 455.

545. Jansen J., Kluin-Nelemans J.C., van Damme J., Wientjens G.J.H.M., Willemze R., Fibbe W.E. (1992) Interleukin 6 is a permissive factor for monocytic colony formation by human hematopoietic progenitor cells. Journal of Experimental Medicine. **175** :1151.

546. Gannasin R.C., Bols N.C. (1998) Development of a monocyte/macrophage-like cell line, RTS11, from rainbow trout spleen. Fish & Shellfish Immunology. **8**: 457.

547. Vallejo A.N., Elllsaesser C.F., Miller N.W., Clem L.W. (1991) Spontaneous development of functionally active long-term monocyte-like cell lines from channel catfish. In vitro Cellular & Developmental Biology. **27(A)**: 279.

548. Miller N.W., Chinchar V.G., Clem L.W. (1994) Development of leukocyte cell lines from channel catfish (*Ictalurus punctatus*). Journal of Tissue Culture Research. **16**: 117.

549. Gannasin R.C., Bols N.C. (1996) Development of long-term rainbow trout spleen cultures that are haematopoietic and produce dendritic cells. Fish & Shellfish Immunology. **6**: 17.

550. Dannevig B.H., Brudeseth B.E., Gojen T., Rode M., Wergeland H.I., Evensen O., Press C.M. (1997) Characterisation of a long-term cell line (SHK-1) developed from the head kidney of Atlantic salmon (*Salmo salar* L.). Fish & Shellfish Immunology. **7**: 213.

551. Weyts F.A.A., Rombout J.H.W.M., Flik G., Verburg-van-Kemenade B.M.L. (1997) A common carp (*Cyprinus carpio* L.) leukocyte cell line shares morphological and functional similarities with macrophages. Fish & Shellfish Immunology. **7**: 123.

552. Barreda D., Belosevic M. (2001) Characterisation of growth enhancing factor production in different phases of *in vitro* fish macrophage development. Fish & Shellfish Immunology. **11**: 169.

553. Joerink M., Saeij J., Stafford J. L., Belosevic M., Wiegertjes G. (2003) Animal models for study of innate immunity: protozoan infections in fish. SEB Experimental Biology Reviews: 55th Edition **(in press).**

554. Verburg-van-Kemenade B.M.L., Daly J.G., Groeneveld A., Wiegertjes G.F. (1996) Multiple regulation of carp (Cyrinus carpio L.): genetic dtermination, isolation, and partial characterisation. Journal of Experimental Biology. **51**: 189.

555. Chilmonczyk S., Monge D. (1999) Flow cytometry as a tool for assessment of the fish cellular immune responses to pathogens. Fish & Shellfish Immunology. **9**: 319.

556. Schmidt J.A., Marshall J., Hayman M.J., Ponka P., Beug H. (1986) Control of erythroid differentiation: possible role of the transferrin cycle. Cell. **46**: 41.

557. Becker S. (1984) Influence of interferon on human monocyte to macrophage development. Cellular Immunology. **84**: 145.

558. Johnson W.D., Mei B.J., Cohn A. (1977) The separation, long-term cultivation and maturation of the human monocye. Journal of Experimental Medicine. **146**: 1613.

559. Zuckerman S.H., Ackerman S.K., Douglas S.D. (1979) Long-term peripheral blood monocyte-macrophage cell cultures. Immunology. **38**: 401.

560. Martin J.H.J., Edwards S.W. (1993) Changes in mechanisms of monocyte/macrophage-mediated cytotoxicity during culture. Journal of Immunological Methods. **150**: 3478.

561. Afonso A., Lousada S., Sliva J., Ellis A.E., Silva M.T. (1998) Neutrophil and macrophage responses to inflammation in the peritoneal cavity of rainbow trout *Oncorhynchus mykiss*. Diseases of Aquatic Organisms. **34**: 27.

562. Lorsbach R.B., Murphy W.J., Lowenstein C.J., Snyder S.H., Russell S.W. (1993) Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. Molecular basis for the synergy between interferon-gamma and lipopolysaccharide. Journal of Biological Chemistry. **268**: 1908.

563. Nathan C. (1991) Modulation of the antimicrobial host immune response: mechanisms and modulation of macrophage activation. Boehring Institute Mitteilungen. **88**: 200.

564. Nacy C.A. (1991) T-cell-mediated activation of macrophages. Current Opinion in Immunology. **3**: 330.

565. Vodovotz T., Bogdan C., Paik J., Xie Q.-W., Nathan C.A. (1993) Mechanisms of suppression of nitric oxide release by transforming growth factor beta. Journal of Experimental Medicine. **178**: 605.

566. Bogdan C., Vodovotz Y., Paik J., Xie Q.-W., Nathan C.A. (1994) Mechanisms of suppression of nitric oxide synthase expression by interleukin-4 in primary mouse macrophages. Journal of Leukocyte Biology. **55**: 227.

567. Bogdan C., Vodovotz Y., Nathan C.A. (1991) Macrophage deactivation by interleukin-10. Journal of Experimental Medicine. **174**: 1549.

568. Heidenreich S., Gong J.-H., Schmidt A., Nain M., Gemsa D. (1989) Macrophage activation by granulocyte/macrophage colony stimulating factor: priming for enhanced release of tumor necrosis factor alpha and prostaglandin E2. Journal of Immunology. **143**: 1198.
569. Lowry M.A.R., Goldberg J.L., Belosevic M. (1998) Induction of nitric oxide (NO) synthesis in murine macrophages requires potassium channel activity. Clinical & Experimental Immunology. **111**: 597.
570. Scott K.D., Stafford J.L., Glavez F., Belosevic M., Goss G.G. (2003) Plasma membrane depolarization reduces nitric oxide (NO) production in P388D.1 macrophage-like cells during *Leishmania major* infection. Cellular Immunolology. **1**: 58.
571. Hille B. (1992) Ionic channel of excitable membranes. Sunderland: Sinauer Associates Inc.
572. Gallin E.K. (1991) Ion channels in leukocytes. Physiological Reviews. **71**: 775.
573. Randriamampita C., Trautmann A. (1987) Ionic channels in murine macrophages. Journal of Cellular Biology. **105**: 761.
574. Schmid-Antomarchi H., Schmid-Alliana A., Romey G., Ventura M.-A., Breittmayer V., Millet M.-A., Husson H., Moghrabi B., Lazdunski M., Rossi B. (1997) Extracellular ATP and UTP control the generation of reactive oxygen intermediates in human macrophages through the opening of charybdtoxin-sensitive Ca2+-dependent K+ channel. Journal of Immunology. **159**: 6209.
575. Maruyama N., Kakuta Y., Yamauchi K., Ohkawara Y., Aizawa T., Ohrui T., Nara M., Oshiro T., Ohno I., Tamura G. (1994) Quinine inhibits production of tumor necrosis factor-alpha from human alveolar macrophages. American Journal of Respiratroy Cell and Molecular Biology **10**: 514.
576. Caggiona A.O., Kraig R.P. (1998) Prostaglandin E2 and 4-aminopyridine prevent lipopolysaccharide-induced outwardly rectifying current and interleukin-1beta production in cultured rat microglia. Journal of Neurochemistry. **70**: 2357.
577. Candy K.G., Gutman G.A., Grissmer S. (1993) Physiological role, molecular structure and evolutionary relationships of voltage gated potassium channels in T lymphocytes. Seminars in Neuroscience. **5**: 125.
578. Schlicter L., Sidell N., Hagiwara S. (1986) Potassium channels mediate killing in human natural killer cells. Proceedings of the National Academy of Sciences of the United States of America. **83**: 451.
579. Stafford J.L., Galvez F., Goss G.G., Belosevic M. (2002) Induction of nitric oxide and respiratory burst response in activated goldfish macrophages requires potassium channel activity. Developmental and Comparative Immunology. **26**: 445.
580. De Kinkelin P., Dorson M. (1973) Interferon production in rainbow trout (*Salmo gairdneri*) experimentally infected with Egtved virus. The Journal of General Virology. **19**: 125.

581. Dorson M., De Kinkelin P., Torchy C. (1992) Interferon synthesis in rainbow trout fry following infection with infectious pancreatic necrosis virus. Fish & Shellfish Immunology. **2**: 311.

582. Congleton J., Sun B. (1996) Interferon-like activity produced by anterior kidney leukocytes of rainbow trout stimulated *in vitro* by infectious hematopoietic necrosis virus or Poly I:C. Diseases of Aquatic Organisms. **25**: 185.

583. Grondel J.L., Harmsen E.G.M. (1984) Phylogeny of interleukins: growth factors produced by leukocytes of the cyprinid fish, *Cyprinus carpio L.* Immunology. **52**: 477.

584. Siegel M.M., Hamby B.A., Huggins E.M.J. (1986) Fish lymphocytes respond to human IL-1 and epithelial cells produce an IL-1 like factor. Veterinary Immunology and Immunopathology. **12**: 47.

585. Elasaesser C.F., Clem L.W. (1994) Functionally distinct high and low molecular weight species of channel catfish and mouse interleukin-1. Cytokine. **5**: 10.

586. Verburg-van-Kemenade B.M.L., Weyts F.A.A., Debets R., Flik G. (1995) Carp macrophages and neutrophilic granulocytes secrete an interleukin-1-like factor. Developmental and Comparative Imunology. **19**: 59.

587. Caspi R.R., Avtalion R.R. (1984) Evidence for the existence of an IL-2-like lymphocyte growth promoting factor in bony fish. Developmental and Comparative Imunology. **8**: 51.

588. Watsin D., Cohen N. (1985) The phylogeny of interleukin-2. Developmental and Comparative Imunology. **9**: 819.

589. Fujiki K., Nakao M., Shin D., Yano T. (1998) cDNA cloning of a carp homologue of mammalian interleukin-1beta. EMBL accession number AB010701.

590. Wang T., Johnson N., Zou J., Bols N., Secombes C.J. (2000) Sequencing of a second interleukin-1beta allele in rainbow trout: analysis of its frequency distribution and expression. EMBL accession number AJ278242.

591. Sumathy K., Kartiki V.D., Kondaiah P. (1997) Isolation of transforming growth factor beta 2 from a fish, *Cyprinus carpio* by RT-PCR. Gene. **191**: 103.

592. Daniels G.D., Secombes C.J. (1999) Genomic organisation of rainbow trout, *Oncorhynchus mykiss* TGF-beta. Developmental and Comparative Imunology. **23**: 139.

593. Daniels G.D., Belosevic M., Secombes C.J. (1997) Partial sequence of goldfish, *Carassius auratus*, transforming growth factor beta. EMBL accession No. AJ001040.

594. Fujiki K., Nakao M., Dixon B. (2003) Molecular cloning and characterisation of a carp (*Cyprinus carpio*) cytokine-like cDNA that shares sequence similarity with IL-6 subfamily cytokines CNTF, OSM and LIF. Developmental and Comparative Immunology. **27**: 127.

595. Tamai T., Shirahata S., Noguichi T., Sata N., Kimura S., Muradami H. (1993) Clonining and expression of flatfish (*Paralichthys olivaceus*) interferon cDNA. Biochemical and Biophysical Acta. **1174**: 182.

596. Altmann S.M., Mellon M.T., Distel D.L., Kim C.H. (2003) Molecular and functional analysis of an interferon gene from zebrafish, *Danio rerio*. Journal of Virology. **77**: 1992.

597. Long S., Wilson M., Bengten E., Bryan L., Clem L.W., Miller N.W., Chinchar V.G. (2003) Identification of a cDNA encoding channel catfish interferon. Developmental and Comparative Immunology. **(in press)**.

598. Ding A., Nathan C.A., Graycar J., Dernck R., Stuehr D.J., Srimal S. (1990) Macrophage deactivating factor and transforming growth factors -beta1, -beta 2, and -beta 3 inhibit induction of macrophage nitric oxide synthesis by IFN-gamma. Journal of Immunology. **145**: 940.

599. Fiorentino D.F., Zlotnik A., Viera P., Mosmann T.R., Howard M., Moore K.W., O'Garra A. (1997) IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. Journal of Immunology. **146**: 3444.

600. Zhan Y., Jimmy K. (2000) Molecular isolation and characterization of carp transforming growth factor beta1 from activated leukocytes. Fish & Shellfish Immunology. **10**: 309.

601. Harms C.A., Kennedy-Stoskopf S., Horne W.A., Fuller F.J., Tompkins W.A.F. (2000) Cloning and sequencing hybrid striped bass (*Morone saxitilis X M. chrysops*) transforming growth factor-beta (TGF-beta), and development of a reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay to measure TGF-beta mRNA of teleost fish. Fish & Shellfish Immunology. **10**: 61.

602. Laing K.J., Cunningham C., Secombes C.J. (2000) Genes for three different isoforms of transforming growth factor-beta are present in plaice (*Pleuronectes platessa*) DNA. Fish & Shellfish Immunology. **10**: 261.

603. Kohli G., Hu S., Clelland E., Muccio R., Rothenstein J., Peng C. (2003) Cloning of transforming growth factor-beta1 (TGF-beta1) and its type II receptor from zebrafish ovary and role of TGF-beta1 in oocyte maturation. Endocrinology. **144**: 1931.

604. Wu C.-C., Thiemermann C., Vane J.R. (1995) Glibenclamide-induced inhibition of the expression of inducible nitric oxide synthase in cultured macrophages and in the anaesthetized rat. British Journal of Pharmacology. **114**: 1273.

605. Eder C. (1998) Ion channels in microglia (brain macrophages). American Journal of Physiology. **275**: C327.

606. Hara N., Ichinose M., Sawach M., Maeno T. (1991) The activation of Ca2+-dependent K+ conductance by adrenaline in mouse macrophages. Pflugers Arch. **419**: 371.

607. Mackinnon R. (1991) New insights into the structure and function of potassium channels. Current Opinion in Neurobiology. **1**: 14.

608. Jan L.Y., Jan Y.N. (1992) Structural elements involved in specific K+ channel functions. Annual Review of Physiology. **54**: 537.

609. Gallin E.K., Sheehy P.A. (1985) Differential expression of inward and outward potassium currents in the macrophage-like cell line J-774.1. Journal of Physiology. **369**: 475.

610. Artalejo C.R., Rossie S., Perlman R.L., Fox A.P. (1992) Voltage-dependent phosphorylation may recruit Ca2+ current facilitation in chromaffin cells. Nature. **358**: 63.

611. Spranger M., Kiprianova I., Krempien S., Schwab S. (1998) Reoxygenation increases the release of reactive oxygen intermediates in murine microglia. Journal of Cerebral Blood Flow and Metabolism. **18**: 670.

612. Hinz B., Brune K., Pahl A. (2000) Nitric oxide inhibits inducible nitric oxide synthase mRNA expression in RAW 264.7 macrophages. Biochemical and Biophysical Research Communications. **271**: 353.

613. Griscavage J.M., Hobbs A.J., Ignarro L.J. (1995) Negative modulation of nitric oxide synthase by nitric oxide and nitroso compounds. Advances in Pharmacology. **34**: 215.

614. Robinson V.K., Sato E., Nelson D.K., Camhi S.L., Robbins R.A., Hoyt J.C. (2001) Peroxynitrite inhibits inducible (type 2) nitric oxide synthase in murine lung epithelial cells in vitro. Free Radical Biology & Medicine. **30**: 986.

615. Blunck R., Scheel O., Muller M., Brandenburg K., Seitzer U., Seydel U. (2001) New insights into endotoxin-induced activation of macrophages: involvement of a K+ channel in transmembrane signaling. Journal of Immunology. **166**: 1009.

616. Haslberger A., Romanin C., Koerber R. (1992) Membrane potential modulates release of tumor necrosis factor in lipopolysaccharide-stimulated mouse macrophages. Molecular Biology of the Cell. **3**: 451.

617. Nathan C.F., Karnovsky M.L., David J.R. (1971) Alterations of macrophage functions by mediators from lymphocytes. Journal of Experimental Medicine. **133**: 1356.

618. Nathan C.F., Remold H.G., David J.R. (1973) Characterization of a lymphocyte factor,which alters macrophage functions. Journal of Experimental Medicine. **1973**: 275.

619. Stuehr D.J., Marletta M.A. (1987) Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon-gamma. Journal of Immunology. **158**: 670.

620. Hardie L.J., Fletcher T.C., Secombes C.J. (1995) Effect of temperature on macrophage activation and the production of macrophage activating factor by rainbow trout (*Oncorhynchus mykiss*) leucocytes. Developmental and Comparative Immunology. **18**: 57.

621. Matzinger P. (1994) Tolerance, danger and the extended family. Annual Review of Immunology. **12**: 991.

622. Matzinger P. (2002) The danger model: a renewed sense of self. Science. **296**: 301-5.

623. Dvorak H.N., Senger D.R., Dvorak M., Harvey V.S., McDonagh J.M. (1985) Regulation of extravascular coagulation by microvascular permeability. Science. **227**: 1059.

624. Furie B., Furie B.C. (1988) The molecular basis of blood coagulation. Cell. **53**: 505.

625. Yamada K.M. (1991) In Cell Biology of Extracellular matrix (Hay, E.D., ed) 2nd Ed., Plenum Publishing Corp., New York 1991: 111.

626. Jarnagin W.R., Rockey D.C., Koteliansky V.E., Wang S.-S., Bissell D.M. (1994) Expression of variant fibronectins in wound healing: cellular source and biological activity if the EIIIA segment in rat hepatic fibrogenesis. Journal of Cellular Biology. **127**: 2037.

627. George J., Wang S.-S., Sevcsik A.M., Sanicola M., Cate R.L., Kotcliansky V.E., Bissell D.M. (2000) Transforming growth factor-beta initiates wound repair in rat liver through induction of the EIIIA-fibronectin splice isoform. American Journal of Pathology. **156**: 115.

628. Saito S., Yamaji N., Yasunaga K., Saito T., Matsumoto S.-I., Katoh M., Kobayashi S., Masuho Y. (1999) The fibronectin exta domain A activates matrix metalloproteinase gene expresion by an interleukin-1-dependent mechanism. Journal of Biological Chemistry. **274**: 30756-63.

629. Hino K., Shiozawa S., Kuroki Y., Ishikawa H., Shiozawa K., Sekiguchi K., Hirano H., Sakashita E., Miyashita K., Chihara K. (1995) EDA-containing fibronectin is synthesized from rheumatoid synovial fibroblast-like cells. Arthritis and Rheumatism. **38**: 678.

630. Yoshiga T., Georgieva T., Dunkov B.C., Harizanova N., Ralchev K., Law J.H. (1999) *Drosophila melanogaster* transferrin. Cloning, deduced protein sequence, expression during the life cycle, gene localization and up-regulation on bacterial infection. European Journal of Biochemistry. **260**: 414.

631. Birch H.E., Schreiber G. (1986) Transcriptional regulation of plasma protein during inflammation. Journal of Biological Chemistry. **261**: 8077.

632. Medzhitov R., Preston-Hurlburt P., Janeway C.A.J. (1997) A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. Nature. **388**: 394.

633. Janeway C.A., Medzhitov R. (1999) Lipoproteins take their Toll on the host. Current Biology. **9**: R879.

634. Medzhitov R., Janeway C.A.J. (1988) Self-defense: the fruit fly style. Proceedings of the National Academy of Sciences of the United States of America. **95**: 429.

635. Govind S., Stewart R. (1991) Dorsoventral pattern formation in *Drosophila*: signal transduction and nuclear targeting. Trends in Genetics. **7**: 119.

636. O'Neill L.A., Greene C. (1998) Signal transduction pathways activated by the IL-1 receptor family: ancient signaling machinery in mammals, insects, and plants. Journal of Leukocyte Biology. **63**: 650.

637. Kato M., Wang H., Kainulainen V., Fitzgerald M.L., Ledbetter S., Ornitz D.M., Bernfield M. (1998) Physiological degradation converts the soluble syndecan-1 ectodomain from an inhibitor to a potent activator of FGF-2. Nature Medicine. **4**: 691.

638. Subramanian S.V., Fitzgerald M.L., Bernfield M. (1997) Regulated shedding of syndecan-1 and -4 ectodomains by thrombin and growth factor receptor activation. Journal of Biological Chemistry. **272**: 14713.

639. Platt J.L., Dalmasso A.P., Lindman B.J., Ihrcke N.C., Bach F.H. (1991) The role of C5a and Ab in the release of heparan sulfate from endothelial cells. European Journal of Immunology. **21**: 2887.

640. Ihrcke N.C., Parker W., Reissner K.J., Platt J.L. (1998) Regulation of platelet heparanase during inflammation: role of pH and proteinases. Journal of Cellular Physiology. **175**: 255.

641. Park P.W., Pier G.B., Preston M.J., Goldberger O., Fitzgerald M.L., Bernfield M. (2000) Syndecan-1 shedding is enhanced by LasA, a secreted virulence factor of *Pseudomonas aeruginosa*. Journal of Biological Chemistry. **275**: 3057.

642. Ihrcke N.C., Platt J.L. (1996) Shedding of heparan sulfate proteoglycan by stimulated endothelial cells: evidence for proteolysis of cell surface molecules. Journal of Cellular Physiology. **168**: 625.

643. Platt J.L., Vercellotti B.J., Lindman B.J., Oegeman T.R., Bach F.H.J. (1990) Release of heparan sulfate from endothelial cells: implications for the pathogenesis of hyperacute rejection. Journal of Experimental Medicine. **171**: 1363.

644. Buczek-Thomas J.A., Nugent M.A. (1999) Elastase-mediated release of heparan sulfate proteoglycans from pulmonary fibroblast cultures: a mechanism for basic fibroblast growth factor (bFGF) release and attenuation of bFGF binding following elastase-induced injury. Journal of Biological Chemistry. **274**: 25167.

645. Key N.S., Platt J.L., Vercellotti B.J. (1992) Vascular endothelial cell proteoglycans are susceptible to cleavage by neutrophils. Arteriosclerosis and Thrombosis. **12**: 836.

646. Klebanoff S.J., Kinsella M.G., Wight T.N. (1993) Degredation of endothelial cell matrix heparan sulfate proteoglycan by elastase and the myeloperoxidase-H2O2-chloride system. The American Journal of Physiology. **143**: 907.

647. Staskawicz B.J., Mudgett M.B., Dangl J.L., Galan J.E. (2001) Common and contrasting themes of plant and animal diseases. Science. **292**: 2285.

648. Reymond P., Kunz B., Paul-Pletzer K., Grimm R., Eckerskorn C., Farmer E.E. (1996) Cloning of a cDNA encoding a plasma membrane-associated, urodine binding phosphoprotein with physical properties similar to viral movement proteins. The Plant Cell. **8**: 2265.

649. O'Neill L.A., Dinarello C.A. (2000) The IL-1 receptor/Toll-like receptor superfamily: crucial receptors for inflammation and host defense. Immunology Today. **21**: 206.

650. Aderem A., Ulevitch R.J. (2000) Toll-like receptors in the induction of the innate immune response. Nature. **406**: 782.

651. Medzhitov R., Janeway C.A.J. (1998) Innate immune recognition and control of adaptive immune responses. Seminars in Immunology. **10**: 351.

652. Chow J.C., Young D.W., Golenbock D.T., Christ W.J., Gusovsky F. (1999) Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. Journal of Biological Chemistry. **274**: 10689.

653. Searle S., Kerr J.F., Bishop C.J. (1982) Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. Pathology Annual. **17**: 229.

654. Regunathan S., Feinstein D.L., Reis D.J. (1999) Anti-proliferative and anti-inflammatory actions of imidazoline agents. Are imidazoline receptors involved? Annals of the New York Academy of Sciences. **21**: 410.

655. Djeha A., Perez-Arellano J.L., Hayes S.L., Brock J.H. (1986) Transferrin synthesis by macrophages: up-regulation by gamma-interferon and effect on lymphocyte proliferation. Journal of Clinical Investigation. **77**: 841.

656. Djeha A., Perez-Arellano J.L., Brock J.H. (1993) Transferrin synthesis by mouse lymph node and peritoneal macrophages: iron content and effect on lymphocyte proliferation. Blood. **81**: 1046.

657. Basu S., Binder R.J., Suto R., Anderson K.M., Srivastava P.K. (2000) Necrotic but not apoptotic cell death releases heat shock proteins, which deliver a partial maturation signal to dendritic cells and activate the NF-kappa B pathway. International Immunology. **12**: 1539.

658. Li M., Carpio D.F., Zheng Y., Bruzzo P., Singh V., Ouaaz F., Medzhitov R.M., Beg A.A. (2001) An essential role of the NF-kappa-beta/Toll-like receptor pathway in induction of inflammatory and tissue-repair gene expression by necrotic cells. Journal of Immunology. **166**: 7128.

659. Saadi S., Wrenshall L.E., Platt J.L. (2002) Regional manifestations and control of the immune system. FASEB Journal. **16**: 849.

660. Matzner Y., Bar-Ner M., Yahalom J., Ishai-Michaeli R., Fuks Z., Vlodavsky I. (1985) Degradation of heparan sulfate in the subendothelial extracellular matrix by a readily released heparanase from human neutrophils. Possible role in invasion through basement membranes. Journal of Clinical Investigation. **76**: 1306.

661. Bashkin P., Razin E., Eldor A., Vlodavsky I. (1990) Degranulating mast cells secrete an endoglycosidase that degrades heparan sulfate in subendothelial extracellular matrix. Blood. **75**: 2204.

662. Naparstek Y., Cohen I.R., Fuks Z., Vlodavsky I. (1984) Activated T lymphocytes produce a matrix-degrading heparan sulphate endoglycosidase. Nature. **310**: 241.

663. Shimada K., Ozawa T. (1987) Subendothelial extracellular-matrix heparan sulfate proteoglycan-degrading activity of human monocyte macrophages. Heart Vessels. **3**: 175.

664. Pfundt R., van Ruissen F., van Vlijmen-Willems I.M.J.J., Alkemade H.A.C., Zeeuwen P.L.J.M., Jap P.H., Dijkman H., Fransen J., Croes H., van Erp P.E.J., Schalkwijk J. (1996) Constitutive and inducible expression of SKALP/Elafin provides anti-elastase defence in human epithelia. Journal of Clinical Investigation. **98**: 1389.

665. Remold-O'Donnell E., Nixon J.C., Rose D.M. (1989) Elastase inhibitor: characterization of the human elastase inhibitor molecule associated with monocytes, macrophages, and neutrophils. Journal of Experimental Medicine. **169**: 1071.

666. Ohbayashi H. (2002) Novel neutrophil elastase inhibitors as a treatment for neutrophil-predominant inflammatory lung disease. IDrugs. **5**: 910.

667. Ligoxygakis P., Pelte N., Hoffmann J.A., Reichhart J.M. (2002) Activation of *Drosophila* Toll during fungal infection by a blood serine protease. Science. **297**: 114.

668. Robertson A.S., Belorgey D., Lilley K.S., Lomas D.A., Gubb D., Dafforn T.R. (2003) Characterization of the necrotic protein that regulates the Toll-mediated immune response in *Drosophila*. Journal of Biological Chemistry. **278**: 6175.

669. Doring G. (1994) The role of neutrophil elastase in chronic inflammation. American Journal of Respiratory and Critical Care Medicine. **150**: S114.

670. Travis J., Pike R., Imamura T., Potempa J. (1994) The role of proteolytic enzymes in the development of pulmonary emphysema and peridontal disease. American Journal of Respiratory and Critical Care Medicine. **150**: S143.

671. Bieth J.G. (1986) Elastases:catalytical and biological properties. In Regulation of matrix accumulation. R. Mecham, editor. Academic Press. New York, NY. 217.

672. Gronski T.J.J., Martin R.L., Kobayashi D.K., Walsh B.C., Holman M.C., Huber M., Van Wart H.E., Shapiro D. (1997) Hydrolysis of a broad spectrum of extracellular matrix proteins by human macrophage elastase. Journal of Biological Chemistry. **272**: 12189.

673. Doring G., Pfestorf M., Botzenhart K., Abdallah M.A. (1988) Impact of proteases on iron uptake of *Pseudomonas aeruginosa* from transferrin and lactoferrin. Infection and Immunity. **56**: 291.

674. Britigan B.E., Hayek M.B., Doebbeling B.N., Fick R.B.J. (1993) Transferrin and lactoferrin undergo proteolytic cleavage in the *Pseudomonas aeruginosa*-infected lungs of patients with cystic fibrosis. Infection and Immunity. **61**: 5049.

675. Britigan B.E., Edeker B.L. (1991) *Pseudomonas* and neutrophil products modify transferrin and lactoferrin to create conditions that favor hydroxyl radical formation. Journal of Clinical Investigation. **88**: 1092.

676. Miller R.A., Britigan B.E. (1995) Protease-cleaved iron-transferrin augments oxidant-mediated endothelial cell injury via hydroxyl radical formation. Journal of Clinical Investigation. **95**: 2491.

677. Miller R.A., Rasmussen G.T., Cox C.D., Britigan B.E. (1996) Protease cleavage of iron-transferrin augments pycocyanin-mediated endothelial cell injury via promotion of hydroxyl radical formation. Infection and Immunity. **64**: 182.

678. Britigan B.E., Rosen G.M., Chai Y., Cohen M.S. (1986) Do human neutrophils make hydroxyl radical? Detection of free radicals generated by human neutrophils activated with a soluble or particulate stimulus using electron paramagnetic resonance spectrometry. Journal of Biological Chemistry. **261**: 4426.

679. Gutteridge J.M.C., Paterson S.K., Segal A.W., Halliwell B. (1981) Inhibition of lipid peroxidation by the iron-binding protein lactoferrin. Biochemical Journal. **199**: 259.

680. Ward P.A., Till G.O., Kunkel R., Beauchamp C. (1983) Evidence for the role of hydroxyl radical in complement and neutrophil-dependent tissue injury. Journal of Clinical Investigation. **72**: 789.

681. Shepherd V.L., Lopez M. (2001) The role of surfactant-assocoated protein A in pulmonary host defense. Immunologic Research. **23**: 111.

682. Crouch E., Wright J.R. (2001) Surfactant proteins A and D and pulmonary host defense. Annual Review of Physiology. **63**: 521.

683. McIntosh J.C., Mervin-Blake S., Conner E., Wright J.R. (1996) Surfactant protein A protects growing cells and reduces TNF-alpha activity from LPS-stimulated macrophages. The American Journal of Physiology. **271**: L310.

684. Sano H., Sohma H., Muta T., Nomura S., Voelker D.R., Kuroki Y. (1999) Pulmonary surfactant protein A modulates the cellular response to smooth and rough lipopolysaccharides by interaction with CD14. Journal of Immunology. **163**: 387.

685. Stamme C., Walsh E., Wright J.R. (2000) Surfactant protien A differentially regulates IFN-gamma- and LPS-induced nitrite production by rat alveolar macrophages. American Journal of Respiratroy Cell and Molecular Biology. **23**: 772.

686. Arias-Diaz J., Garcia-Verdugo I., Casals C., Sanchez-Rico N., Vara E., Balibrea J.L. (2000) Effect of surfactant protein A (SP-A) on the production of cytokines by human pulmonary macrophages. Shock. **14**: 300.

687. Phelps D.S. (2001) Surfactant regulation of host defense function in the lung: a question of balance. Pediatric Pathology & Molecular Medicine. **20**: 269.

688. Griese M. (1999) Pulmonary surfactant in health and human lung disease: state of the art. The European Respiratroy Journal. **13**: 1455.

689. Ikeda R.A., Bowman B.H., Yang F., Lokey L.K. (1992) Production of human serum transferrin in *Escherichia coli*. Gene. **117**: 265.

690. Steinlein L.M., Ikeda R.A. (1993) Production of N-terminal and C-terminal human serum transferrin in *Escherichia coli*. Enzyme and Microbial Technology. **15**: 193.

691. Mason A.B., Miller M.K., Funk W.D., Banfield D.K., Savage K.J., Oliver R.W., Green B.N., MacGillivray R.T., Woodworth R.C. (1993) Expression of glycosylated and nonglycosylated human transferrin in mammalian cells. Characterization of the recombinant proteins with comparison to three commercially available transferrins. Biochemistry. **32**: 5472.

692. Mason A.B., He Q.-Y., Adams T.E., Gumerov D.R., Kaltashov I.A., Nguyen V., MacGillivray R.T.A. (2002) Expression, purification, and characterization of recombinant nonglycosylated human serum transferrin containing a C-terminal hexahistidine tag. Protein expression and purification. **23**: 142.

693. Steinlein L.M., Ligman C.M., Kessler S., Ikeda R.A. (1998) Iron release is reduced by mutations of lysines 206 and 296 in recombinant N-terminal half transferrin. Biochemistry. **37**: 13696.

694. Ali S.A., Joao H.C., Hammerschmid F., Eder J., Steinkasserer A. (1999) Transferrin trojan horses as a rational approach for the biological delivery of therapeutic peptide domains. Journal of Biological Chemistry. **274**: 24066.

695. Rock F.L., Hardiman G., Timans J.C., Kastelein R.A., Bazan J.F. (1998) A family of human receptors structurally related to *Drosophila* Toll. Proceedings of the National Academy of Sciences of the United States of America. **95**: 588.

696. Fukui A., Inoue N., Matsumoto M., Nomura M., Yamada K., Matsuda Y. *et al*. (2001) Molecular cloning and functional characterization of chicken Toll-like receptors. A single chicken Toll covers multiple molecular patterns. Journal of Biological Chemistry. **276**: 143.

697. Kobe B,, Deisenhofer J. (1994) The leucine-rich repeat: a versatile binding motif. Trends in Biochemical Sciences. **19**: 415.

698. Kobe B., Deisenhofer J. (1995) A structural basis of the interactions between leucine-rich repeats and protein ligands. Nature. **374**: 183.

699. Hausmann M., Kiessling S., Mestermann S., Webb G., Spottl T., Andus T.*et al. (*2002) Toll-like receptors 2 and 4 are up-regulated during intestinal inflammation. Gastroenterology. **122**: 1987.

700. Cario E., Podolsky D.K. (2000) Differential alteration in intestinal epithelial cell expression of Toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease. Infection and Immunity. **68**: 7010.

701. Mita Y., Dobashi K., Endou K., Kawata T., Shimizu Y., Nakazawa T.*et al.* (2002) Toll-like receptor 4 surface expression on human monocytes and B cells is modulated by IL-2 and IL-4. Immunology Letters. **81**: 71.

702. Sato S., Nomura F., Kawai T., Takeuchi O., Muhlradt P.F., Takeda K.e.a. (2000) Synergy and cross-tolerance between Toll-like receptor (TLR) 2- and TLR4-mediated signaling pathways. Journal of Immunology. **165**: 7096.

703. Nomura F., Akashi S., Sakao Y., Sato S., Kawai T., Matsumoto M.*et al.* (2000) Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface Toll-like receptor 4 expression. Journal of Immunology. **164**: 3476.

704. Youssef H.A., Sigurdsson G.H., Christenson J.T., Owunwanne A. (1991) Use of indium-111-labeled transferrin to study plasma extravasation during endotoxin shock and the effects of the beta-2 agonist terbutaline. Aerican Journal of Physiologic Imaging. **6**: 85.

705. Grinnell F., Zhu M. (1996) Fibronectin degradation in chronic wounds depends on the relative levels of elastase, alpha1-proteinase inhibitor and alpha2-macroglobulin. The Journal of Investigative Dermatology. **106**: 335.

706. Schmidtchen A. (2000) Degradation of antiproteinases, complement and fibronectin in chronic leg ulcers. Acta Dermato-Venereologica. **80**: 179.

707. Agren M.S., Eaglstein W.H., Ferguson M.W., Harding K.G., Moore K., Saarialho-Kere U.K., Schultz G.S. (2000) Causes and effects of the chronic inflammation in venous leg ulcers. Acta Dermato-Venereologica. **210**: 8.

708. Rao C.N., Ladin D.A., Liu Y.Y., Chilukuri K., Hou Z.Z., Woodley D.T.J. (1995) Alpha 1-antitrypsin is degraded and non functional in chronic wounds but intact and functional in acute wounds: the inhibitor protects fibronectin from degradation by chronic wound fluid enzymes. The Journal of Investigative Dermatology. **105**: 572.

709. Schmidtchen A., Holst E., Tapper H., Bjorck L. (2003) Elastase-producing *Pseudomonas aeruginosa* degrade plasma proteins and extracellular products of human skin and fibroblasts, and inhibit fibroblast growth. Microbial Pathogenesis. **34**: 47.

710. Zhang H., Patel S.A., Kandil E., Mueller C.M., Lin Y.Y., Zenilman M.E. (2003) Pancreatic elastase is proven to be a mannose-binding protein--implications for the systemic response to pancreatitis. Surgery. **133**: 678.

711. Wakabayashi H., Takase M., Tomita M. (2003) Lactoferricin derived from milk protein lactoferrin. Current Pharmaceutical Design. **9**: 1277.

712. Eliassen L.T., Berge G., Sveinbjornsson B., Svendsen J.S., Vorland L.H., Rekdal O. (2002) Evidence for a direct antitumor mechanism action of bovine lactoferrin. Anticancer Research. **22**: 2703.

713. Morisato D., Anderson K.V. (1994) A processed form of the Spatzle protein defines dorsal-ventral polarity in the *Drosophila* embryo. Cell. **76**: 677.

714. Matsuguci T., Musikacharoen T., Ogawa T., Yoshikai Y. (2000) Gene expressions of Toll-like receptor 2, but not Toll-like receptor 4, is induced by LPS and inflammatory cytokines in mouse macrophages. Journal of Immunology. **165**: 5767.

715. An H., Xu H., Yu Y., Zhang M., Qi R., Yan X., *et al.,* (2002) Up-regulation of TLR9 gene expression by LPS in mouse macrophages via activation of NF-kappaB, ERK and p38 MAPK signal pathways. Immunology Letters. **81**: 165.

716. Biragyn A., Ruffini P. A., Leifer C. A., Klyushnenkova E., Shakhov A., Chertov O., Shirakawa A. K., *et al.,* (2002) Toll-like receptor 4-dependent activation of dendritic cells by β-defensin 2. Science. **298**: 1025.